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Research Article

# A new luciferase immunoprecipitation system assay provided serological evidence for missed diagnosis of severe fever with thrombocytopenia syndrome

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ABSTRACT

Severe fever with thrombocytopenia syndrome (SFTS), caused by SFTS virus (SFTSV) infection, was first reported in 2010 in China with an initial fatality of up to 30%. The laboratory confirmation of SFTSV infection in terms of detection of viral RNA or antibody levels is critical for SFTS diagnosis and therapy. In this study, a new luciferase immunoprecipitation system (LIPS) assay based on pREN2 plasmid expressing SFTSV *NP* gene and tagged with Renilla luciferase (Rluc), was established and used to investigate the levels of antibody responses to SFTSV. Totally 464 serum samples from febrile patients were collected in the hospital of Shaoxing City in Zhejiang Province in 2019. The results showed that 82 of the 464 patients (17.7%) had antibody response to SFTSV, which were further supported by immunofluorescence assays (IFAs). Further, qRT-PCR and microneutralization tests showed that among the 82 positive cases, 15 patients had viremia, 10 patients had neutralizing antibody, and one had both (totally 26 patient). However, none of these patients were diagnosed as SFTS in the hospital probably because of their mild symptoms or subclinical manifestations. All the results indicated that at least the 26 patients having viremia or neutralizing antibody were the missed diagnosis of SFTS cases. The findings suggested the occurrence of SFTS and the SFTS incidence were higher than the reported level in Shaoxing in 2019, and that LIPS may provide an alternative strategy to confirm SFTSV infection in the laboratory.

## 1. Introduction

The Severe fever with thrombocytopenia syndrome (SFTS) disease caused by SFTS virus (SFTSV) infection was first reported in China in 2010 with an initial fatality rate of up to 30% (Yu et al., 2011). The major clinical signs of SFTS include high fever and severe thrombocytopenia, accompanied with gastrointestinal symptoms, respiratory symptoms, neurologic symptoms, and hemorrhagic manifestations (Xiong et al., 2016). As of 2019, 25 provinces in China have reported over 13,000 SFTS cases to the National Notifiable Disease Reporting System including the laboratory-confirmed and probable cases. The seven provinces locating in the Eastern and Central China including Henan, Shandong, Anhui, Hubei, Liaoning, Zhejiang, and Jiangsu reported 99.3% of the total cases, with the highest laboratory confirmation rates of 100% occurring in Zhejiang and Jiangsu (Huang et al., 2021).

SFTSV belongs to the genus *Bandavirus* in the family *Phenuiviridae*, the genome of which is composed of three RNA segments (*S*, *M* and *L*). The *S* segment encodes nucleoprotein (NP) and non-structure protein NSs; the *M* segment encodes glycoprotein (GP) which would be cleaved into Gc and Gn proteins during maturation process of viral particles; and the *L* segment encodes RNA-depend RNA polymerase (RdRp) which is critical for RNA transcription. Identification of SFTSV infections is critical for the diagnosis of SFTS. According to guidelines issued in 2010 by the National Health Commission of the People's Republic of China for the prevention

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and treatment of SFTS (http://www.gov.cn/gzdt/2010-10/09/content_1718261.htm), SFTSV infections can be laboratory confirmed using reverse transcription polymerase chain reaction (RT-PCR) to amplify SFTSV RNA or via subculture of patient serum samples to isolate virus. The guidelines also stated that detection of serological response specific to SFTSV is also an effective method to identify SFTSV infection. Seroconverted patients with anti-SFTSV IgG levels or neutralization activity four times higher than those during the acute stage are considered to have a recent SFTSV infection. The presence of total anti-SFTSV antibodies detected using double-antigen sandwich ELISA can also be used to confirm SFTSV exposure.

In this study, a new method for serological assay to determine the levels of total antibodies against SFTSV was established based on the luciferase immunoprecipitation system (LIPS). LIPS is a fluid-phase immunoassay which has been used for measuring antoantibodies in a wide range of aoutoimmune studies and antibodies against infectious agents including fungi, bacteria, and virus (Burbelo et al., 2015). This assay was applied to investigate the serological response to SFTSV among the febrile patients in Shaoxing City located in Zhejiang Province in 2019. Moreover, the epidemic features were characterized according to the results of LIPS assay and other laboratory tests using the patients' serum samples. The results revealed the occurrence of SFTSV infection in Shaoxing and suggested the presence of missed diagnosis of SFTS cases probably due to the subclinical manifestations. Our findings further suggested that LIPS assay could be an alternative strategy to identify SFTSV infection in laboratory.

## 2. Materials and methods

### 2.1. Cell, virus, and antibodies

African green monkey kidney (Vero) cells (ATCC, CCL-81, Lot#: 60150897), Human embryonic kidney (HEK293T) cells (ATCC, CRL-11268, Lot#: 62296864) were purchased from the American type culture collection and were cultured by using Dulbecco's Modified Eagle's Medium-High glucose (DMEM, NZK biotech, Wuhan, China) with 10% fetal bovine serum (FBS, Gibco, Australia). SFTSV strain HBGS13 was isolated from the serum sample of an SFTS patient in Hubei Province (Zhang et al., 2017) and was preserved in National Virus Resource Center (IVCAS 6.6312, NVRC, Wuhan, China).

The rabbit polyclonal antibodies against SFTSV nucleoprotein (NP) (anti-SFTSV NP) were prepared in the laboratory (Zhang et al., 2017; Shen et al., 2018). The Goat anti-Human IgG H&L (FITC) (abcam, Shanghai, China), the Goat anti-Human IgM H&L (FITC) (abcam), and the Goat anti-Rabbit IgG H&L (Alexa Fluor ®488) (abcam) were used as the secondary antibodies in IFA tests. The anti-FLAG Tag antibody (Sangon Biotech, Shanghai, China) was used as primary antibody in verifying expression of LIPS-related fusion protein. The Goat anti-Rabbit IgG (H + L) conjugated with horseradish peroxidase (HRP) (Proteintech, Wuhan, China) was used as the secondary antibody to verify expression of LIPS-related fusion protein.

### 2.2. Collection of serum samples from febrile patients and healthy persons

A total of 464 serum samples from febrile patients (≥(37.3 °C) in Shaoxing City, Zhejiang Province were collected during April to June (223 samples numbered from A1 to A223) and August to October (241 samples numbered from B1 to B241) in Shaoxing People's Hospital in 2019. Infection with HIV, HBV, HCV and epidemic hemorrhagic fever-related pathogens was excluded by nosocomial tests. The personal information of these febrile patients including gender, age, and clinical parameters was summarized as shown in Table 1. The serum samples from 10 febrile patients positive for Tamdy virus (TAMV, genus *Orthonairovirus* in family *Nairoviridae*) antibody test (Moming et al., 2021), were archived in National Virus Resource Center and used as negative control. Serum samples from 30 healthy donors aged from 22 to 69, with the median age of 29 (Table 1), were collected in Wuhan City in 2019 and used as healthy control.

**Table 1**
Personal information, clinical parameters, and results of laboratory tests from the febrile patients and healthy persons in this study.

| Characteristics | | Febrile patients (n = 464) | Healthy Donors (n = 30) |
|---|---|---|---|
| Personal information | | | |
| Gender | Male (n, %) | 251, 54.1% | 15, 50% |
| | Female (n, %) | 213, 45.9% | 15, 50% |
| Age, Median (IQR) | | 65 (54–76) | 29 (25–35) |
| **Clinical manifestation** | | | |
| Fever | | 464, 100% | 0 |
| PLT (100–300×$10^9$/L), Median (IQR)[a] | | 182 (134–226) | N/A |
| WBC (4–10×$10^9$/L), Median (IQR)[a] | | 6.03 (4.68–9.71) | N/A |
| **Laboratory Findings** | | **Positive rate (n, %)** | |
| Viremia | | 16, 3.4% | N/A |
| LIPS Test (anti-NP) | | 82, 18.7% | 0 |
| MNT[b] | | 11, 2.4% | N/A |

IQR, interquartile range; PLT means platelet; WB, white blood cell; MNT, microneutralization test.

[a] Clinical indices (PLT and WBC) of the 80 LIPS-positive patients were collected.

[b] All LIPS-Anti-SFTSV-NP positive samples were tested by MNT assay.

### 2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA was purified from the human serum sample (100 μL for each sample) by using TRIzol (NZK biotech, Wuhan, China) according to the manufacture's instruction, then subpackaged and deposited at −80 °C until further experiments. The viral RNA loads were determined by qRT-PCR as previously described (Zhu et al., 2019) by using the commercial one step TB Green® PrimeScript™ PLUS RT-PCR kit (Perfect Real Time) (TAKARA, Japan).

### 2.4. Immunofluorescence assay (IFA) and Western blot

The plasmid transfected HEK 293T cells or SFTSV-infected Vero cells were fixed with 4% paraformaldehyde about 15 min, and permeabilized by 0.2% Triton-X 100 for 15 min at room temperature. After three washes with phosphate buffer saline (PBS, pH = 7.2), cells was blocked in PBS containing 5% (w/v) bovine serum albumin (BSA, BioFrox, Germany) at 37 °C for 1 h. Then, cells were incubated with human serum samples (1:20 dilution in PBS containing 1% BSA), the anti-SFTSV NP polyclonal antibody (1:1000), or the anti-Flag tag antibody (1:1000) as primary antibodies at 4 °C overnight. After three washes, cells were incubated with the Goat anti-Human IgG H&L (FITC), the Goat anti-Human IgM H&L (FITC) and the Goat anti-Rabbit IgG H&L (Alexa Fluor ® 488) respectively, according to manufacturer's instruction. Finally, fluorescence was visualized and images were taken using an inverted fluorescence microscope.

Western blot was used to verify expression of SFTSV NP in fusion with Flag-tag in the plasmid-transfected cells. The anti-FLAG tag or anti-SFTSV-NP rabbit polyclonal antibody (1:1000 dilution) were used as the primary antibody, HRP-conjugated goat anti-Rabbit IgG (H + L) (1:3000 dilution) were used as the secondary antibody.

### 2.5. Luciferase immunoprecipitation system (LIPS)

The SFTSV *NP* gene (GenBank No: JQ341190) was cloned into the pREN2 plasmid (kindly provided by Prof. Linfa Wang and Prof. Peng Zhou as previously described (Uehara et al., 2019)) in-frame with the Renilla luciferase (Rluc) and Flag-tag by using the ClonExpress®Ultra One Step Cloning Kit (Vazyme, Nanjing, China) according to manufacturer's instruction and was verified by Sanger sequencing. The plasmids were transfected into HEK293T cells using Lipofectamine™ 3000

Transfection Reagent (Invitrogen, America). At 48 h post transfection, cells were harvested and lysed for luciferase assay directly or stored at −80 °C until further experiments. Luciferase assays were performed as previously described (Burbelo et al., 2019). Briefly, transfected cells were lysed on ice for 20 min, followed by cell disruption using an Ultrasound cell disruptor (Sonics, America) for 3 min. The lysis was diluted and prepared for each test having $10^7$ light units (LU) per 10 μL. Serum samples were 1:50 diluted in Buffer A (20 mmol/L Tris, pH 7.5; 150 mmol/L NaCl; 5 mmol/L $MgCl_2$; 1% Triton X 100). For the LIPS test of each sample, a mixture of 90 μL diluted serum/plasma sample, 10 μL cell lysis containing viral antigen, and 7 μL 30% Pierce Protein A/G UltraLink Resin beads (Thermo Fisher Scientific, Waltham, MA) was prepared and thoroughly blended at 4 °C for 2 h. Then, beads were washed six times with Buffer A and two times with phosphate buffered solution (PBS). The light units of each test were measured using Renilla-Lumi™ Plus Luciferase Assay Kit (Beyotime, Shanghai, China). The cut-off value of light units was set as the average value of control samples plus triple its standard deviation. Febrile patients tested positive for SFTSV antibody by LIPS was listed in Supplementary Table S1.

### 2.6. Microneutralization tests against SFTSV

Patient serum samples were serially diluted (from 1:8 to 1:128) in DMEM containing 2% FBS and incubated with 100 $TCID_{50}$ SFSTV for each test at 37 °C for 1 h. The virus-serum mixture was added to Vero cells ($10^4$ cells/well) seeded in the 96-well plates and incubated at 37 °C for 72 h. IFAs were performed to visualize SFTSV infection in each well. The neutralization titers were expressed as the reciprocal of the highest dilution that prevents SFTSV infection in three replicates.

### 2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism (Version 8, San Diego, California) and IBM SPSS Statistics (Version 19, Chicago). *P* values less than 0.05 were considered statistically significant.

## 3. Result

### 3.1. LIPS assay was established based on SFTSV-NP expression

Serological responses to SFTSV were investigated in all 464 patients by LIPS assay. The method was established based on the expression of SFTSV nucleoprotein (NP), and was validated by both IFAs and Western blot which detected the successful expression of SFTSV NP in fusion with Flag-tag in the plasmid-transfected cells (Fig. 1A and B). Subsequently, serial dilutions of the polyclonal rabbit antibody against anti-SFTSV NP were used to demonstrate the feasibility of LIPS to determine antibody levels. The amount of Renilla luciferase (Rluc)-NP-antibody complex captured by protein A/G in each test was measured and presented in the form of Rluc light units (LU). As expected, the dilutions containing decreased levels of anti-SFTSV NP antibody binding to SFTSV NP was detected in a concentration-dependent manner (Fig. 1C).

### 3.2. SFTSV infection was identified from a few of febrile patients in Shaoxing

SFTSV infection was suspected among the 464 febrile patients from Shaoxing City, including 251 men (54.1%) and 213 women (45.9%), especially among those samples collected during the peak season of tick activity. Demographic and clinical characteristics of the 464 febrile patients were summarized in Table 1. The median age of these patients is 65 with the interquartile range (IQR) from 54 to 76. The counts of platelets (PLT) and white blood cells (WBC) of 80 patients were obtained from the cases identified with serological response to SFTSV by LIPS, the median and IQR of which were at respective normal ranges of PLT and WBC. Serum samples from the 223 febrile patients collected from April to June were used to detect SFTSV infection by qRT-PCR. The results showed that 16 (3.4%) of the 464 samples were positive for SFTSV RNA (Table 1), with the virus load ranging from $1.13 \times 10^4$ to $1.83 \times 10^7$ copies/mL in sera (Table 2), which suggested the 16 patients had SFTSV viremia and revealed the occurrence of SFTSV infection in Shaoxing. Detection of the other 241 samples for SFTSV RNA was not performed due to the sample limits.

### 3.3. Positive serological response to SFTSV was found among the febrile patients in Shaoxing City

Antibody responses to SFTSV were subsequently investigated in the febrile patient serum samples using LIPS. In total, 82 (17.7%) cases of the 464 patients had a positive serological response to SFTSV (Table 1), including 47 male (10.1%) and 35 female (7.5%) (Supplementary Table S1). Fifty-six patients (12.1%) were >60 years of age, suggesting a significantly high SFTS incidence among the elderly ($P < 0.001$) (Supplementary Table S1). In addition, the specificity of this method was

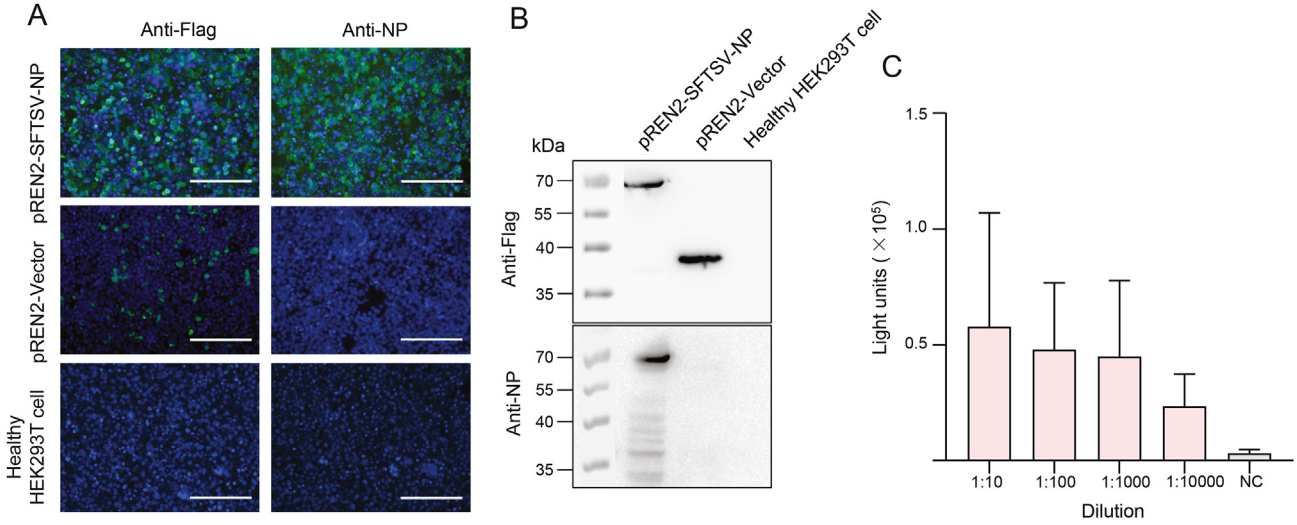


**Fig. 1.** LIPS assay was established, validated and further used for antibody detection in serum samples from febrile patients. Expression of SFTSV NP in fusion with Flag tag in pREN2 plasmid transfected HEK293T cells was confirmed by IFAs (Scale bar = 400 μm) (**A**) and Western blot (**B**), respectively. (**C**) LIPS was used to detect the rabbit polyclonal antibody against SFTSV NP in different dilutions. Results were shown as the Rluc light units (LU). Tests were performed in triplicates. Bars, standard deviations; NC, negative control by using the un-inoculated rabbit serum.

**Table 2**
Detailed results of laboratory tests with serum samples from the LIPS-positive patients and viral RNA-positive patients, including virus RNA copies, antibody levels, and neutralizaing antibody titers, as well as the clinical diagnosis and parameters of routine blood tests.

| Time of collection | Sample | Viral loads[a] (copies/mL) | Anti SFTSV | | | MNT (Titer)[d] | Diagnosis[e] | Other pathogen testing[f] | Leukopenia[g] | Thrombocytopenia[h] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | LIPS[b] | IFA-IgM[c] | IFA-IgG[c] | | | | | |
| April to June | **A4** | **-** | **10.09** | + | **-** | **$2^4$** | **Infectious fever** | – | | |
| | A10 | – | 5.09 | + | + | – | Digestive disease | – | | |
| | **A20** | **-** | **2.88** | **-** | + | **$2^3$** | **N/A** | **N/A** | | |
| | **A27** | **$2.71 \times 10^6$** | **1.39** | + | + | **-** | **Respiratory disease** | **-** | **$2.78 \times 10^9$/L** | **$107 \times 10^9$/L** |
| | **A30** | **$8.28 \times 10^6$** | **1.05** | + | + | **-** | **Respiratory disease** | **Influenza B+** | **$3.32 \times 10^9$/L** | |
| | A31 | – | 7.36 | + | + | – | Infectious fever | Influenza A+ | $2.55 \times 10^9$/L | $94 \times 10^9$/L |
| | A39 | – | 2.32 | + | + | – | N/A | N/A | | |
| | A48 | – | 3.25 | + | + | – | Respiratory disease | Parainfluenza virus+ | | |
| | A50 | – | 1.06 | – | + | – | Respiratory disease | – | | |
| | **A54** | **$4.98 \times 10^6$** | **1.09** | **-** | + | **-** | **Fever of unknown origin** | **-** | | |
| | **A57** | **$9.51 \times 10^6$** | **1.52** | + | **-** | **$2^4$** | **Other disease** | **-** | | |
| | A70 | – | 1.19 | + | – | – | N/A | – | | |
| | A73 | – | 1.07 | – | + | – | Infectious fever | – | | $78 \times 10^9$/L |
| | A77 | – | 7.20 | + | – | – | Infectious fever | Influenza A+ | | $71 \times 10^9$/L |
| | A81 | – | 13.47 | + | – | – | Infection fever | – | $2.63 \times 10^9$/L | |
| | A82 | – | 6.47 | – | + | – | Other disease | – | | |
| | A88 | – | 1.01 | + | + | – | Infection fever | – | $3.29 \times 10^9$/L | |
| | A89 | – | 9.28 | – | – | – | Other disease | – | | |
| | A94 | – | 1.47 | + | + | – | Other disease | – | | |
| | **A97** | **-** | **18.13** | **-** | + | **$2^3$** | **Respiratory disease** | **-** | | |
| | A113 | – | 1.25 | – | + | – | Respiratory disease | – | | |
| | A117 | – | 25.05 | – | + | – | Other disease | – | | |
| | A119 | – | 4.24 | + | + | – | Severe thrombocytopenia | – | | $68 \times 10^9$/L |
| | **A128** | **-** | **13.19** | **-** | + | **$2^4$** | **Respiratory disease** | **-** | | |
| | **A134** | **-** | **1.31** | **-** | + | **$2^4$** | **Other disease** | **-** | | |
| | A139 | – | 1.36 | + | + | – | N/A | N/A | | |
| | A141 | – | 1.28 | – | + | – | N/A | N/A | | |
| | A146 | – | 2.66 | – | + | – | N/A | N/A | | |
| | A147 | – | 1.37 | – | + | – | N/A | N/A | | |
| | A148 | – | 1.36 | – | + | – | Respiratory disease | Influenza A+ | | |
| | A153 | – | 1.41 | – | + | – | N/A | N/A | | |
| | A154 | – | 1.43 | – | + | – | N/A | N/A | | |
| | **A155** | **-** | **38.05** | + | + | **$2^5$** | **Hemophagocytic syndrome** | **-** | **$2.25 \times 10^9$/L** | **$68 \times 10^9$/L** |
| | A165 | – | 1.01 | – | – | – | Infectious fever | – | $2.38 \times 10^9$/L | |
| | **A169** | **-** | **14.58** | **-** | + | **$2^3$** | **N/A** | **N/A** | | |
| | A174 | – | 3.24 | – | + | – | N/A | N/A | | |
| | A177 | – | 1.74 | – | + | – | N/A | N/A | | |
| | A181 | – | 12.19 | – | + | – | N/A | N/A | | |
| | A185 | – | 3.99 | – | – | – | N/A | N/A | | |
| | A192 | – | 2.95 | + | + | – | N/A | N/A | | |
| | A195 | – | 1.02 | + | + | – | N/A | N/A | | |
| | A197 | – | 12.98 | + | – | – | N/A | N/A | | |
| | A198 | – | 1.22 | + | + | – | N/A | N/A | | |
| | A199 | – | 1.63 | + | + | – | Respiratory disease | – | | |
| | **A209** | **$2.94 \times 10^6$** | **1.86** | + | **-** | **-** | **Respiratory disease** | **-** | | |
| | A210 | – | 1.00 | – | – | – | N/A | N/A | | |
| | A211 | – | 2.61 | – | – | – | Infection fever | – | | $96 \times 10^9$/L |
| | A214 | – | 6.35 | – | + | – | N/A | N/A | | |
| | **A219** | **-** | **1.26** | + | + | **$2^4$** | **N/A** | **N/A** | | |
| | **A6** | **$2.08 \times 10^6$** | **<1** | **N/A** | **N/A** | **N/A** | **Respiratory disease** | **-** | | |
| | **A18** | **$2.57 \times 10^6$** | **<1** | **N/A** | **N/A** | **N/A** | **N/A** | **N/A** | | |
| | **A29** | **$4.01 \times 10^6$** | **<1** | **N/A** | **N/A** | **N/A** | **Other disease** | **-** | | |
| | **A37** | **$3.37 \times 10^6$** | **<1** | **N/A** | **N/A** | **N/A** | **Other disease** | **-** | | |
| | **A41** | **$4.01 \times 10^6$** | **<1** | **N/A** | **N/A** | **N/A** | **Fever of unknown origin** | **-** | **$3.3 \times 10^9$/L** | |
| | **A65** | **$4.88 \times 10^6$** | **<1** | **N/A** | **N/A** | **N/A** | **Heart disease** | **-** | | |
| | **A104** | **$1.83 \times 10^7$** | **<1** | **N/A** | **N/A** | **N/A** | **Infectious fever** | **-** | | |
| | **A130** | **$3.53 \times 10^4$** | **<1** | **N/A** | **N/A** | **N/A** | **Heart disease** | **-** | | |
| | **A160** | **$2.74 \times 10^4$** | **<1** | **N/A** | **N/A** | **N/A** | **Respiratory disease** | **-** | | |
| | **A162** | **$1.13 \times 10^4$** | **<1** | **N/A** | **N/A** | **N/A** | **Severe thrombocytopenia** | **-** | **$4.08 \times 10^9$/L** | **$73 \times 10^9$/L** |
| | **A187** | **$3.44 \times 10^6$** | **<1** | **N/A** | **N/A** | **N/A** | **Respiratory disease** | **-** | | |
| Subtotal | **60** | **16** | **49** | **23** | **37** | **9** | | | **9** | **8** |
| August to October | B6 | N/A | 2.49 | – | + | – | Infectious fever | – | $1.23 \times 10^9$/L | |
| | B9 | N/A | 1.34 | – | + | – | Respiratory disease | – | $3.89 \times 10^9$/L | $96 \times 10^9$/L |
| | B12 | N/A | 5.15 | + | + | – | Respiratory disease | – | | |

(*continued on next page*)

**Table 2** (*continued*)

| Time of collection | Sample | Viral loads[a] (copies/mL) | Anti SFTSV | | | MNT (Titer)[d] | Diagnosis[e] | Other pathogen testing[f] | Leukopenia[g] | Thrombocytopenia[h] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | LIPS[b] | IFA-IgM[c] | IFA-IgG[c] | | | | | |
| | B21 | N/A | 1.03 | + | – | – | N/A | N/A | | |
| | B24 | N/A | 1.43 | – | + | – | Respiratory disease | – | | |
| | B26 | N/A | 1.59 | + | + | – | Infectious fever | – | | $89 \times 10^9$/L |
| | B29 | N/A | 2.00 | + | + | – | respiratory disease | – | $2.49 \times 10^9$/L | |
| | B31 | N/A | 1.11 | – | + | – | Other disease | – | | |
| | **B37** | **N/A** | **3.99** | **+** | **-** | **$2^5$** | **Respiratory disease** | **Dengue virus+** | **$1.38 \times 10^9$/L** | |
| | B44 | N/A | 2.48 | + | + | – | Respiratory disease | – | | |
| | B45 | N/A | 3.93 | + | – | – | Respiratory disease | – | | $85 \times 10^9$/L |
| | B47 | N/A | 1.08 | + | – | – | Respiratory disease | – | | |
| | B54 | N/A | 1.33 | – | + | – | Respiratory disease | – | $1.23 \times 10^9$/L | |
| | B61 | N/A | 2.70 | + | + | – | Respiratory disease | – | | |
| | B62 | N/A | 1.48 | – | + | – | Respiratory disease | – | | |
| | B68 | N/A | 1.52 | – | + | – | Other disease | – | | |
| | **B74** | **N/A** | **15.52** | **+** | **-** | **$2^5$** | **Infectious fever** | **-** | | |
| | B79 | N/A | 10.52 | – | – | – | Neurological and digestive disease | – | | |
| | B87 | N/A | 12.46 | – | – | – | Respiratory disease | – | | |
| | B93 | N/A | 9.33 | – | – | – | Other disease | – | | |
| | B112 | N/A | 5.81 | – | – | – | Respiratory disease | – | | |
| | B117 | N/A | 3.23 | + | – | – | Infectious fever | – | | |
| | B142 | N/A | 1.51 | – | – | – | Respiratory disease | – | | |
| | B147 | N/A | 11.62 | + | – | – | Other disease | – | | |
| | B150 | N/A | 1.80 | + | + | – | Other disease | – | | |
| | B183 | N/A | 1.23 | – | + | – | Respiratory disease | – | | |
| | B190 | N/A | 1.85 | – | – | – | Infectious fever | – | | $87 \times 10^9$/L |
| | B206 | N/A | 1.01 | – | – | – | Respiratory disease | – | | |
| | B210 | N/A | 4.11 | – | – | – | Infectious fever | – | | |
| | B212 | N/A | 1.53 | – | + | – | Other disease | – | | |
| | B215 | N/A | 1.37 | – | + | – | Respiratory disease | – | | |
| | B221 | N/A | 5.15 | – | – | – | Infectious fever | – | $3.93 \times 10^9$/L | |
| | B242 | N/A | 2.84 | – | + | – | Respiratory disease | – | | |
| Subtotal | **33** | **N/A** | **33** | **13** | **17** | **2** | | | **6** | **4** |
| Total | **93** | **16** | **82** | **36** | **54** | **11** | | | **15** | **12** |

IFA, immunofluorescence assay; MNT, microneutralization tests; +, positive; -, negative; N/A, not applicable. The 26 cases having viremia and/or neutralization to SFTSV were highlighted in bold font.

[a] qRT-PCR was not performed to the samples collected from Aug to Oct due to sample limitations.

[b] The samples positive for anti-SFTSV antibodies as determined by LIPS were shown as the Light units (LU) fold changes above the cut-off value, while those negative were shown as "<1 ".

[c] IgM and IgG response to SFTSV were detected by IFAs, which were not carried out for the LIPS-negative samples.

[d] Neutralization tests were not performed for the LIPS-negative samples.

[e] The diagnostic records available from 70 patients were listed.

[f] Records of other pathogen testing including blood culture testing, HIV, HBV, HCV, *Treponema pallidum*, Influenza A, Influenza B and epidemic hemorrhagic fever related pathogens.

[g] The normal range of WBC counts in healthy population is from $4 \times 10^9$ to $10 \times 10^9$/L. The counts less than the lower limit were shown, which may be considered leukopenia. The counts within the normal range were not listed.

[h] The normal range of PLT counts in healthy population is from $100 \times 10^9$ to $300 \times 10^9$/L. The counts less than the lower limit were shown, which may be considered thrombocytopenia. The counts within the normal range were not listed.

validated by using serum samples from 10 febrile patients confirmed with antibody response to TAMV. The results showed that these samples did not have significant reaction with SFTSV antigen. Thirty serum samples from healthy donors were also included as healthy control (Fig. 2). These findings were further supported by the IFA results involving 82 LIPS-positive serum samples, 10 randomly selected LIPS-negative samples, and samples from two healthy donors. Sixty-eight of the 82 LIPS-positive serum samples tested positive for anti-SFTSV by IFAs, a confirmation rate of 82.9%. The IFA-positive samples included 14 samples with an IgM only response, 32 with IgG, and 22 with both (Table 2, Supplementary Fig. S1). As expected, the IFA results were negative for the LIPS-negative samples and healthy donor samples (Supplementary Fig. S1). Moreover, In microneutralization tests, 11 of the 82 LIPS-positive serum samples including seven man and four women, had neutralizing antibodies to SFTSV with the neutralizing titers ranging from $2^3$ to $2^5$ (Tables 1 and 2). These results further suggest that SFTSV infections had occurred in Shaoxing in 2019, despite the limited number of collected febrile patients.

### *3.4. Epidemic characteristics and records of clinical diagnosis suggested missed cases of SFTS*

The epidemiologic characteristics with respected to age, gender, underlying disease, days post illness onset, and sampling time were analyzed based on the LU values of each sample, which could represent the levels of total antibodies against SFTSV (Table 3). The patients of 21–40 years old and 41–60, and those aged over 60 had comparable levels of antibody response to SFTSV. This was also observed among the male and female patients. Since 12.1% patients were over 60 years old, their comorbidities may affect the levels of antibody response. Of the 60 LIPS-positive cases, 27 cases had underlying diseases, including hypertension (8, 29.7%), chronic respiratory diseases (9, 33.3%), coronary heart disease (2, 7.4%), metabolic diseases (1, 3.7%), and others (7, 25.9%) (Table 3). The patients with comorbidities had levels of antibody against SFTSV significantly lower than those without ($P = 0.0113$). The antibody levels could change during different time of sample collection post illness onset (Infectious time). However, this change was not

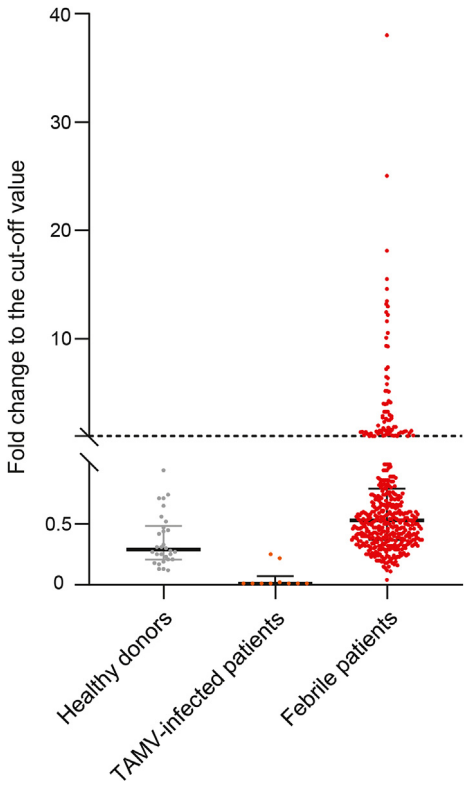


**Fig. 2.** Characteristics of the antibody responses among LIPS-positive cases. Detection of anti-SFTSV NP antibodies in ten TAMV-infected patients and eighty-two febrile patients using a LIPS assay. Thirty serum from healthy donors were tested as healthy control. Antibody levels are shown as fold changes compared with that of the cut-off LU values.

**Table 3**
Analysis of the correlations of gender, age, comorbidities, days post illness onset, and sampling time with levels of antibodies against SFTSV.

| Factor | Antibody levels | *P* value |
|---|---|---|
| | Median ( $\times 10^4$, IQR) | |
| Gender | | |
| Male (n = 47) | 10.8 (5.80–20.1) | 0.9554 |
| Female (n = 35) | 8.32 (5.65–32.8) | |
| Age | | |
| 21–40 (n = 13) | 11.0 (5.69–41.8) | 0.6961 |
| 41–60 (n = 13) | 9.62 (5.15–26.8) | |
| ≥61 (n = 56) | 6.22 (5.88–12.2) | |
| Comorbidities [a] | | |
| Yes (n = 27) | 6.84 (5.42–12.4) | 0.0113 |
| No (n = 33) | 19.0 (7.09–41.8) | |
| Days post illness onset | | |
| 1–7 (n = 46) | 12.7 (8.73–26.9) | 0.8436 |
| 8–14 (n = 8) | 12.6 (8.83–26.7) | |
| 15–21 (n = 12) | 12.4 (6.96–35.4) | |
| > 21 (n = 10) | 19.2 (10.8–48.5) | |
| Month of sampling | | |
| April to June (n = 45) | 15.4 (10.4–61.0) | 0.0007 |
| August to October (n = 20) | 8.45 (6.08–18.3) | |

IQR, interquartile range.
[a] Among the LIPS-positive group, 60 cases had comorbidities including hypertension, chronic respiratory diseases, coronary heart disease, metabolic diseases, and endocrine diseases.

significant among these samples during the first three weeks in this study, which is probably due to the sample limit, whereas the levels slightly increased in the samples collected three weeks after disease onset. Moreover, we found that the LIPS-positive samples collected during April to June, which is the peak season of tick activity, had antibody levels significantly higher than those collected during August to October ($P = 0.0007$) (Table 3). This suggested a potential relationship between the risk of SFTSV infection and tick activity and a high incidence of SFTS during April to June in Shaoxing City. SFTSV neutralizing activity may be associated with antibody levels. Eight patients (A4, A20, A97, A128, A155, A169, B37, and B74) with neutralizing antibodies had high total antibody levels (>2-fold above the cut-off value), despite the correlation was not observed in the significant manner (Table 2).

The diagnostic records of 70 patients were reviewed, including 16 patients with viremia and 60 LIPS-positive patients (Table 2). None of these patients were diagnosed with SFTS, although twenty-two patients had leukopenia, thrombocytopenia, or both (22/70, 31.4%). These patients were mostly diagnosed with respiratory diseases (31/70, 44.3%), followed by infectious fever or fever of unknown origin (17/70, 24.3%), heart diseases (2/70, 2.9%), digestive diseases (2/70, 2.9%), severe thrombocytopenia (2/70, 2.9%), neurological diseases (1/70, 1.4%), and hemophagocytic syndrome (1/70, 1.4%). The other 14 patients (19.7%) were diagnosed with other diseases. Therefore, we speculate that there could be missed SFTS cases among these febrile patients according to the above results of laboratory tests.

## 4. Discussion

SFTS epidemics remain an important public health threat in Zhejiang Province, as for the top fifth high incidence of SFTS and the higher average case fatality rate than other provinces in China (Sun et al., 2017; Wu et al., 2020). The epidemic features of SFTS in Zhejiang from 2011 to 2016 showed that the number of SFTS cases and affected counties increased year by year, with over 50% of the cases reported from April to June and 13.8% of the cases from August to October (Sun et al., 2017). During 2011–2018, there were 37 counties in Zhejiang reporting SFTS cases, about 74% of which were from seven counties locating in Zhoushan City (Daishan), Taizhou City (Linhai, Tiantai, and Sanmen), Ningbo City (Ninghai and Xiangshan), and Hangzhou City (Chun'an), respectively. Shaoxing City, located in the eastern area of Zhejiang Province, was not included in any of the top eleven counties with the highest total incidence of SFTS (Wu et al., 2020). Moreover, from 2011 to 2018, very a few cases (<10) were reported from Shaoxing (only from Shangyu District) according to the records in China Information System for Diseases Control and Prevention (Wu et al., 2020). These data suggest that Shaoxing was not the major area affected by SFTS.

According to the guidelines issued by the National Health Commission of the People's Republic of China, SFTSV infection could be confirmed in terms of identification of viral RNA and antibody response in laboratory, which is important for the clinical diagnosis. LIPS assay is known to be highly sensitive for evaluating total antibody responses to specific antigens. It has been primarily developed for the diagnosis of autoimmune disease (Maeda et al., 2017) and later expanded for the laboratory detection of serological responses to parasites (Matsuu et al., 2017) and viral pathogens, such as Pteropine orthoreovirus (Uehara et al., 2019) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Chia et al., 2020). LIPS was developed for serological tests for SFTSV in Japan recently (Matsumoto et al., 2018; Matsuu et al., 2021). It was firstly established on the basis of glycoprotein expression to detect antibody against SFTSV among over 3000 blood donors in an SFTS endemic area in Japan. However, no positive cases were identified, probably because, on one hand, all tested samples were from healthy blood donors who were unlikely to be exposed to SFTSV, and on the other hand, the results might be affected by the use of viral antigen (Matsumoto et al., 2018). The recent study reported the establishment of LIPS based on SFTSV-NP, which was used to investigate the seroprevalence of SFTSV among animals in Japan. The results showed sero-positive rates of anti-SFTSV antibody for cats, dog, wild boars and deer were 1.9%, 9.6%, 53.9%, and 34.7%, respectively, suggesting a high risk of SFTSV exposure among animals (Matsuu et al., 2021).

Here in this study, we identified cases having infection of SFTSV or exposure to SFTSV among the febrile patients in 2019. In total, 82 (17.1%) of the 464 patients were found having antibody response to SFTSV antigen with LIPS assay, which was further confirmed by IFAs with a positive rate of 82.9% (68/82). This difference could be attributed to the use of protein A/G by LIPS to capture the antibody-SFTSV NP complex, which could recognize almost all types of human antibodies, including all IgG subclasses, IgA, IgE, IgM, and IgD to a lesser extent (Eliasson et al., 1989; Burbelo et al., 2010), whereas the antibody reaction to viral antigen could only be distinguished in separate tests of IFA by using anti-IgM or -IgG secondary antibody. So, LIPS assay may be more sensitive than IFAs, as the LU values from each test reflect the levels of total antibody response. LIPS is also sensitive for detecting various animal antibodies (Burbelo et al., 2011; Matsuu et al., 2021). This was also demonstrated by the tests for the serial dilutions of rabbit polyclonal antibody anti-SFTSV-NP in the current study. Moreover, the LIPS results revealed the antibody reaction specificity to SFTSV, as serum samples from patients having antibody against TAMV did not show cross-reaction with SFTSV. Despite infection of influenza virus (4, 5.7%), parainfluenza virus (1, 1.4%), and dengue virus (1, 1.4%) were found from 6 of the 70 patients with available diagnostic records, they were confirmed having SFTSV infection as evidenced by the results from qRT-PCR, IFA, or neutralization assay (Table 2). Therefore, together with the results from previous studies (Matsumoto et al., 2018; Matsuu et al., 2021), LIPS could be developed to be an effective method in laboratory for the survey of total serological response to SFTSV among humans and animals. In addition, the results by LIPS revealed that there had been substantial occurrence of SFTS in Shaoxing in 2019 and indicated threats to public health, although it is not a major epidemic area of SFTS.

Moreover, the results of qRT-PCR and neutralization assays showed that at least 26 cases had SFTSV infection or exposure to SFTSV, including 15 patients with viremia, 10 patients with neutralizing antibodies, and one with both (Table 2). However, none of these febrile patients were diagnosed SFTS, which could be attributed to their mild manifestations. Not all of SFTSV patients would have the typical symptoms like leukopenia and thrombocytopenia. Among 286 confirmed SFTS cases from eastern China, 75.5% cases had leukopenia and 66.8% cases had thrombocytopenia (Li et al., 2017). A recent study reported an estimated rate of missed SFTS diagnosis at 8.3%, suggesting a higher incidence of SFTS than that currently reported (Huang et al., 2018). The study also suggested that missed cases could be identified based on high IgG titers and IgM response to SFTSV (Huang et al., 2018). Therefore, we speculate that at least the 26 cases were miss-diagnosed of SFTS probably due to their subclinical manifestation of the SFTS symptoms. According to the serological evidence resulted from LIPS assay, the other 56 patients also had exposure to SFTSV. The use of serological detection methods may promote a more accurate diagnosis and laboratory confirmation of SFTS.

In summary, the current study established a LIPS assay based on SFTSV NP expression for serological detection of anti-SFTSV antibodies and determined the levels of antibody response in febrile patients. The results demonstrated the occurrence of SFTSV infection in patients from Shaoxing City of Zhejiang Province and suggested a higher incidence of SFTS than that previously reported. The findings further demonstrated that LIPS may provide an alternative strategy to confirm SFTSV infection in the laboratory.

## Data availability

All the data generated during the current study are included in the article.

## Ethics statement

The study was reviewed and approved for ethical consideration by the institutional review board (IRB) of Wuhan Institute of Virology, CAS (Approval Number: WIVH33202102). All patients enrolled in the study were from Shaoxing People's Hospital. Witten informed consent was provided by all participants.

## Author contributions

Shengyao Chen: conceptualization, formal analysis, investigation, data curation, visualization, writing - original draft, writing - review & editing. Minjun Xu: data curation, investigation, methodology. Xiaoli Wu: data curation, visualization, methodology. Yuan Bai: data curation, investigation. Junming Shi: data curation. Min Zhou: methodology, resources. Qiaoli Wu: methodology, resources. Shuang Tang: methodology. Fei Deng: conceptualization, funding acquisition, project administration, resources, supervision, writing - review & editing. Bo Qin: conceptualization, resources, data curation. Shu Shen: conceptualization, resources, funding acquisition, project administration, supervision, writing - original draft, writing - review & editing.

## Conflict of interest

The authors declare that they have no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.01.018.

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