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# Severe Fever with Thrombocytopenia Syndrome Complicated by Co-infection with Spotted Fever Group Rickettsiae, China

## Technical Appendix

## Methods

### SFTSV Detection and Quantitation by Real-time PCR

Serial anti-coagulated blood and serum samples were collected from clinically suspected patients, at entry into the hospital and during their hospitalization. Viral RNA was isolated from serum samples using QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD, USA), according to the manufacturer's instructions. One-step Primer Script RT-PCR Kit (TaKaRa Bio dalian Inc., Japan) was used according to the manufacturer's instructions for SFTSV detection in a volume of 20 µL containing 10 µL of One Step RT-PCR Buffer (2×), 0.4 µL of TaKaRa Ex Taq HS (5 U/µL) and 0.4 µL of PrimeScript RT Enzyme MixII (TaKaRa Bio dalian Inc., Japan), 1 µL of PCR primer mix (20 µM of sense and antisense each) and 0.5µL of Probe (10 µM), total RNA 2 µL and RNase free $dH_2O$ (5.7 µL). PCR was carried with one cycle of 42°C for 5 min and 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 55°C for 20 s in a LightCycler Real Time PCR apparatus (Roche Diagnostics, Mannheim, Germany). The real-time PCR primers and probe were targeted at the S-segment of the SFTSV. The sequences were as follows: forward:TTCACAGCAGCATGGAGAGG; reverse: GATGCCTTCACCAAGACTATCAATG;

Probe: AACTTCTGTCTTGCTGGCTCCGC. Nested RT-PCR and sequencing of the M-segment were performed on randomly selected positive samples to verify the real-time RT-PCR

results. We have adopted several strategies to prevent contamination. All negative extraction controls were included to detect cross-contamination events within each extraction. To rule out contamination of the PCR reagents or cross-contamination of the PCR plate, water was included as a control, and RNA from the original specimens was capped before the addition of any positive control materials.

Quantitation of virus was performed using quantitative RT-PCR targeting the same gene segments. Real-time PCR was performed in a volume of 20 μL by use of the One-step Primer Script RT-PCR Kit (TaKaRa Bio dalian Inc., Japan) in the CFX96 instrument (Bio-Rad, California, USA). Standard curves or absolute RNA quantification were included in every assay and were generated by using RNA transcripts produced by in vitro transcription of cDNA that included the real time qPCR assay amplicons. Standard curves included five dilutions and three replicate wells for each dilution. All samples were quantified in at least duplicate wells. Levels of SFTSV RNA concentrations were expressed as copies/mL.

#### SFTSV-specific IgG antibody detection by ELISA

SFTSV-specific IgG antibodies were detected by enzyme-linked immunosorbent assay (ELISA) using the same protocol described previously (*1*). Briefly, 96-well polystyrene microtiter plates were coated with purified SFTSV antigen. Serially diluted serum samples starting with 1:10 were added, and after incubation and washing, antihuman IgG conjugates were added. Substrate solution was added for color development. The optical density at 450 nm (A450) of each well was measured. The cutoff limit was 0.1 plus the optical density of the negative control, according to the manufacturer's instructions.

#### Determination of serum cytokine/chemokine concentration

Serum levels of 27 cytokines and chemokines were measured with the use of Bio-plex Pro Human 27-plex cytokine panel (Bio-plex Pro Human 27-plex cytokine panel, Bio-Rad, CA, USA). The tested cytokines included: interleukin (IL)-1β, IL-1 receptor antagonist (IL-1RA), IL-2, IL-4,

IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), basic FGF, interferon (IFN)-γ, IFN-γ-inducible protein (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein 1a (MIP-1α), macrophage inflammatory protein 1β (MIP-1β), platelet-derived growth factor (PDGF-BB), regulated on activation and normally T-cell expressed (RANTES), tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF). Following manufacturer's instruction, a multiplex-biometric immunoassay containing fluorescent microspheres conjugating with monoclonal antibodies specific for target cytokines was performed to test serum cytokine levels. Cytokine concentrations were calculated from standard curves of known concentrations of recombinant human cytokines. Levels were analyzed using Bio-Plex Manager 6.0 software.

#### Data Collection

We performed a chart review of all recruited patients, collected information on demographic characteristics, symptoms and signs, clinical laboratory test results and treatment regimens, etc. These data were drawn from the medical database by a group of trained physicians using a standardized format and entered into an EpiData database. The data were further reviewed for accuracy and consistency by a group of epidemiologists.

#### Main Outcome Measurements

The main outcome measurements, including hemorrhagic manifestations, presence of plasma leakage, neurologic manifestations, respiratory manifestations and death, were consecutively reviewed during the whole hospitalization. The hemorrhage manifestations were defined by presence of petechiae, ecchymosis, hemoptysis, epistaxis, hematuria, hematemesis, melena, vaginal, and gingival bleeding. Plasma leakage was determined by the presence of pleural and/or ascitic fluid or haemoconcentration. Deaths were classified based on clinical diagnoses of the proximate cause of death by the physician.

### Statistical Analyses

Categorical variables were assessed using the $\chi^2$ test or the Fisher exact test. Continuous variables were compared by Student's t-test and assessed with the nonparametric Mann-Whitney U test in case of skewed variables. The comparisons of laboratory parameters every 2 days from disease onset to discharge were performed by generalized estimating equation (GEE) with adjustment for the variables of age, sex and the intervals from disease onset to hospital admission. All analyses were performed by SAS software, version 9.1.3 (SAS Institute, Cary, North Carolina, USA). All p values were 2-tailed and $p<0.05$ was considered statistically significant.

## Results

In total 1080 laboratory-confirmed SFTS patients were hospitalized during the study period, among them 823 patients who had convalescence serum samples collected were recruited into the study. Of the 823 patients, 25 (3.0%) patients were negative for SFTSV RT-PCR and had seroconversion (n = 18) or a 4-fold increase (n = 7) in titers of IgG antibody (Technical Appendix Table 1). Other 798 patients were positive for SFTSV RT-PCR assay. Seventy-seven (8.5%) of the 823 patients provided serologic evidence of SFGR infection according to seroconversion (n = 45) or 4-fold increase of antibody titer (n = 32), composing as the SFTSV-SFGR coinfection group (Technical Appendix Table 2).

### Reference

1. Yu XJ, Liang MF, Zhang SY, Liu Y, Li JD, Sun YL, et al. Fever with thrombocytopenia associated with a novel bunyavirus in China. N Engl J Med. 2011;364:1523–32. PubMed http://dx.doi.org/10.1056/NEJMoa1010095

**Technical Appendix Table 1.** The titers of IgG antibody in the SFTS patients with RT-PCR negative result tested by ELISA

| Patient No. | Titer of IgG antibody at acute stage | Titer of IgG antibody at recovery stage |
|---|---|---|
| 1 | 1:10 | 1:1280 |
| 2 | <1:10 | 1:640 |
| 3 | <1:10 | 1:640 |
| 4 | <1:10 | 1:10 |
| 5 | 1:20 | 1:160 |
| 6 | <1:10 | 1:640 |
| 7 | <1:10 | 1:80 |
| 8 | <1:10 | 1:20 |
| 9 | <1:10 | 1:20 |
| 10 | <1:10 | 1:10 |
| 11 | <1:10 | 1:10 |
| 12 | 1:10 | 1:40 |
| 13 | <1:10 | 1:10 |
| 14 | <1:10 | 1:80 |
| 15 | <1:10 | 1:20 |
| 16 | 1:10 | 1:80 |
| 17 | <1:10 | 1:10 |
| 18 | 1:80 | 1:320 |
| 19 | 1:10 | 1:320 |
| 20 | 1:10 | 1:320 |
| 21 | <1:10 | 1:40 |
| 22 | <1:10 | 1:10 |
| 23 | <1:10 | 1:160 |
| 24 | <1:10 | 1:10 |
| 25 | <1:10 | 1:20 |

**Technical Appendix Table 2.** The titers of IgG antibody for spotted fever group rickettsiae tested by IFA

| Patient No. | Titer of IgG antibody at acute stage | Titer of IgG antibody at recovery stage |
|---|---|---|
| 3 | <1:64 | 1:256 |
| 4 | <1:64 | 1:128 |
| 9 | 1:64 | 1:512 |
| 16 | <1:64 | 1:512 |
| 18 | <1:64 | 1:256 |
| 26 | <1:64 | 1:64 |
| 27 | <1:64 | 1:64 |
| 28 | <1:64 | 1:64 |
| 29 | <1:64 | 1:64 |
| 30 | <1:64 | 1:64 |
| 31 | 1:64 | 1:256 |
| 32 | 1:64 | 1:256 |
| 33 | <1:64 | 1:64 |
| 34 | <1:64 | 1:128 |
| 35 | <1:64 | 1:128 |
| 36 | <1:64 | 1:128 |
| 37 | <1:64 | 1:128 |
| 38 | <1:64 | 1:128 |
| 39 | <1:64 | 1:128 |
| 40 | <1:64 | 1:128 |
| 41 | <1:64 | 1:128 |
| 42 | <1:64 | 1:128 |
| 43 | <1:64 | 1:128 |
| 44 | <1:64 | 1:256 |
| 45 | <1:64 | 1:256 |
| 46 | <1:64 | 1:256 |
| 47 | <1:64 | 1:256 |
| 48 | <1:64 | 1:128 |
| 49 | <1:64 | 1:128 |
| 50 | <1:64 | 1:512 |
| 51 | <1:64 | 1:512 |
| 52 | <1:64 | 1:128 |
| 53 | 1:64 | 1:512 |
| 54 | 1:64 | 1:256 |
| 55 | 1:64 | 1:256 |
| 56 | 1:64 | 1:256 |

| Patient No. | Titer of IgG antibody at acute stage | Titer of IgG antibody at recovery stage |
|---|---|---|
| 57 | 1:64 | 1:512 |
| 58 | 1:64 | 1:512 |
| 59 | 1:64 | 1:256 |
| 60 | 1:64 | 1:512 |
| 61 | 1:64 | 1:256 |
| 62 | 1:64 | 1:1024 |
| 63 | 1:64 | 1:256 |
| 64 | 1:64 | 1:512 |
| 65 | 1:64 | 1:256 |
| 66 | 1:64 | 1:512 |
| 67 | 1:64 | 1:1024 |
| 68 | 1:64 | 1:512 |
| 69 | 1:64 | 1:512 |
| 70 | <1:64 | 1:512 |
| 71 | <1:64 | 1:256 |
| 72 | <1:64 | 1:256 |
| 73 | <1:64 | 1:256 |
| 74 | <1:64 | 1:256 |
| 75 | <1:64 | 1:256 |
| 76 | <1:64 | 1:256 |
| 77 | 1:64 | 1:256 |
| 78 | 1:64 | 1:256 |
| 79 | 1:64 | 1:256 |
| 80 | <1:64 | 1:128 |
| 81 | <1:64 | 1:128 |
| 82 | <1:64 | 1:128 |
| 83 | <1:64 | 1:128 |
| 84 | <1:64 | 1:128 |
| 85 | <1:64 | 1:128 |
| 86 | <1:64 | 1:128 |
| 87 | <1:64 | 1:128 |
| 88 | <1:64 | 1:128 |
| 89 | <1:64 | 1:64 |
| 90 | <1:64 | 1:64 |
| 91 | <1:64 | 1:64 |
| 92 | <1:64 | 1:64 |
| 93 | <1:64 | 1:64 |
| 94 | <1:64 | 1:64 |

| Patient No. | Titer of IgG antibody at acute stage | Titer of IgG antibody at recovery stage |
|---|---|---|
| 95 | <1:64 | 1:64 |
| 96 | <1:64 | 1:64 |
| 97 | <1:64 | 1:64 |

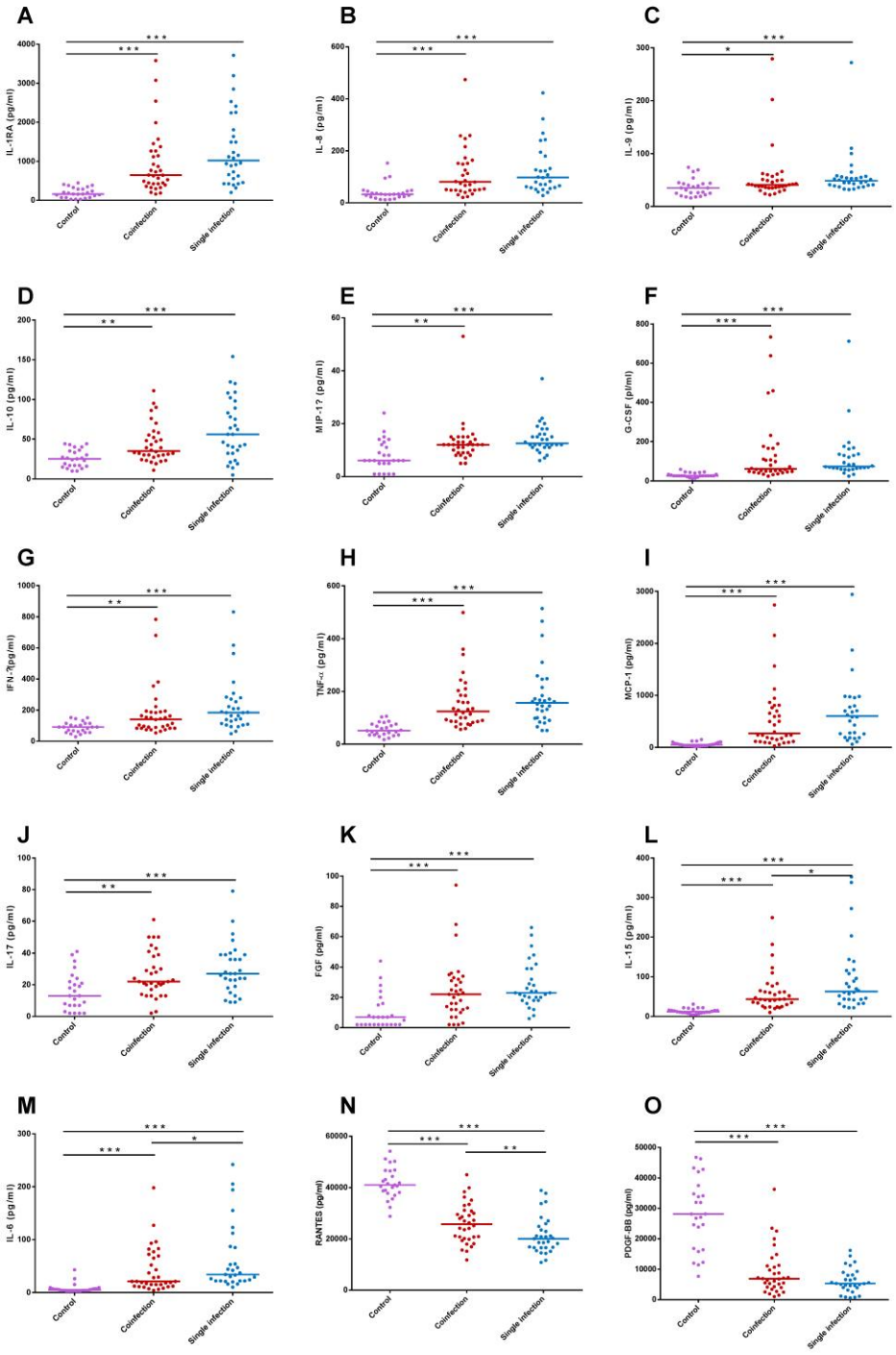
**Technical Appendix Table 3**. Demographic and clinical characteristics of the patients with SFTSV-SFG rickettsiae co-infection *vs.* SFTSV single infection

| Characteristics | Co-infection (n = 77) | Single infection (n = 746) | *P* value |
|---|---|---|---|
| Demographic characteristics | | | |
| Age, mean ± SD, years | 61.4 ± 12.6 | 61.8 ± 12.2 | 0.809 |
| Sex, female, n (%) | 42 (54.6) | 442 (59.3) | 0.425 |
| Hospital duration, median (IQR) | 8(5–10) | 7 (5–9) | 0.137 |
| Delay days from onset to admission, median (IQR) | 5 (4–7) | 5 (4–7) | 0.917 |
| Days>5 from disease onset, n (%) | 38 (49.4) | 355 (47.6) | 0.768 |
| Tick bite history, n (%) | 10 (13.0) | 87 (11.7) | 0.731 |
| Underlying diseases, n (%) | 32 (41.6) | 244 (32.7) | 0.016 |
| Cardiovascular and cerebrovascular diseases | 11 (14.3) | 75 (10.1) | 0.248 |
| Diabetes | 18 (23.4) | 144 (19.3) | 0.392 |
| Active hepatitis | 7 (9.1) | 46 (6.2) | 0.320 |
| Chronic respiratory disease | 2 (2.6) | 14 (1.9) | 0.663 |
| Clinical manifestations | | | |
| Influenza like illness | 77 (100) | 746 (100) | 1.000 |
| Febrile, n (%) | 76 (98.7) | 744 (99.7) | 0.256 |
| Fever duration, days, median (IQR) | 7 (5–9) | 6 (4–8) | 0.039 |
| Lymphadenectasis, n (%) | 33 (42.9) | 334 (44.8) | 0.748 |
| Myalgia, n (%) | 57 (74.0) | 608 (81.5) | 0.113 |
| Weakness, n (%) | 73 (94.8) | 722 (96.8) | 0.362 |
| Dizziness, n (%) | 16 (20.8) | 121 (16.2) | 0.307 |
| Headache, n (%) | 10 (13.0) | 54 (7.2) | 0.073 |
| Chills, n (%) | 7 (9.1) | 60 (8.0) | 0.749 |
| Arthralgia, n (%) | 2 (2.6) | 10 (1.3) | 0.381 |
| Gastrointestinal illness, n (%) | 56(96.1) | 509 (68.2) | 0.418 |
| Abdominal pain | 3 (3.9) | 25 (3.4) | 0.802 |
| Nausea | 53 (68.8) | 469 (62.9) | 0.398 |
| Diarrhea | 20 (26.0) | 146 (19.6) | 0.183 |
| Vomit | 24 (35.2) | 196 (26.3) | 0.848 |
| Diarrhea and Vomit | 36 (46.8) | 288 (38.6) | 0.164 |
| Plasma leakage, n (%) | 9 (11.7) | 70 (9.4) | 0.513 |
| Hydrothorax | 8 (10.4) | 64 (8.6) | 0.592 |

| Characteristics | Co-infection (n = 77) | Single infection (n = 746) | *P* value |
|---|---|---|---|
| Hydropericardium | 1 (1.3) | 12 (1.6) | 0.836 |
| Ascites | 3 (3.9) | 4 (0.5) | 0.002 |
| Respiratory syndrome, n (%) | 34 (44.2) | 377 (50.5) | 0.286 |
| Dyspnea | 5 (6.5) | 4 (2.7) | 0.172 |
| Sputum | 22 (28.6) | 264 (35.4) | 0.232 |
| Cough | 33 (42.9) | 355 (47.6) | 0.429 |
| Hemorrhage manifestations, n (%) | 38 (49.4) | 244 (32.7) | 0.003 |
| Melena | 8 (10.4) | 34 (4.6) | 0.027 |
| Gingival bleeding | 9 (11.7) | 60 (8.0) | 0.272 |
| Hemoptysis | 2 (2.6) | 15 (2.0) | 0.730 |
| Haematemesis | 2 (2.6) | 29 (3.9) | 0.571 |
| Hematuria | 13 (16.9) | 63 (8.5) | 0.074 |
| Petechial/ecchymosis | 20 (26.0) | 119 (16.0) | 0.025 |
| Epistaxis | 0 (0) | 7 (0.9) | 0.393 |
| Neurologic symptom, n (%) | 29 (37.7) | 243 (32.6) | 0.366 |
| Coma | 1 (1.3) | 50 (6.7) | 0.061 |
| Dysphoria | 11 (14.3) | 71 (9.5) | 0.184 |
| Lethargy | 1 (1.3) | 40 (5.4) | 0.690 |
| Blurred mind | 13 (16.9) | 133 (17.8) | 0.836 |
| Convulsion | 11 (14.3) | 99 (13.3) | 0.803 |
| Apathy | 15 (19.5) | 143 (19.2) | 0.947 |

**Technical Appendix Table 4.** The association between death and the related variables

| | Univariate | | Multivariate | |
|---|---|---|---|---|
| Variables | OR (95% CI) | *P* | OR (95% CI) | *P* |
| Model 1 in all the patients | | | | |
| Group (coinfection/single) | 1.845 (0.970–3.508) | 0.062 | 1.992 (1.025–3.873) | 0.042 |
| Age | 1.061 (1.036–1.087) | <0.001 | 1.061 (1.036–1.867) | <0.001 |
| Sex (male/female) | 1.882 (1.203–2.945) | 0.006 | 1.716 (1.082–2.720) | 0.022 |
| Interval from onset to admission | 1.142 (1.055–1.235) | 0.001 | 1.107 (1.020–1.202) | 0.015 |
| Model 2 in the coinfection group | | | | |
| Age | 1.082 (1.014–1.155) | 0.017 | 1.109 (1.023–1.202) | 0.012 |
| Sex (male/female) | 9.167 (1.870–44.922) | 0.006 | 12.491 (1.977–78.910) | 0.007 |
| Interval from onset to admission | 0.757 (0.160–4.963) | 0.895 | 0.860 (0.585–1.265) | 0.443 |
| Model 3 in SFTSV single group | | | | |
| Age | 1.063 (1.037–1.090) | <0.001 | 1.057 (1.030–1.084) | <0.001 |
| Sex (male/female) | 1.517 (0.938–2.454) | 0.090 | 1.314 (0.799–2.160) | 0.282 |
| Interval from onset to admission | 1.182 (1.086–1.286) | <0.001 | 1.142 (1.047–1.247) | 0.003 |




**Technical Appendix Figure.** Comparison of cytokine production in two groups of patients. *p<0 0.05, **p<0 0.01, ***p<0 0.001. Altogether 34 coinfected patients, 30 age and gender comparable SFTS patients and 25 healthy controls who showed no positive SFTSV or *R. rickettsii* detection by either molecular or antibody tests were measured. The 27 cytokines were identified with different patterns of production.

Cytokines IL1-RA, IL-8, IL-9, IL-10, IFN-γ, MCP-1α, G-CSF, FGF, TNF-α, MCP-1 and IL-17 were significantly elevated in two groups than controls, yet showing no inter-group difference (Figure, panels A–K). Cytokine IL-6 and IL-15 were elevated in both groups, with SFTSV single infection attaining significantly higher level than the coinfection group (Figure, panels L, M). PDGF-BB and RANTES was decreased in both groups (Figure, panels N, O), and showing inter-group difference for RANTES only (Figure, panel N). The remaining cytokines were within the normal level and displayed no significant difference between two groups. Abbreviations: G-CSF, granulocyte colony-stimulating factor; IL, interleukin; RANTES, regulated on activation and normally T-cell expressed. PDGF: platelet-derived growth factor.