J Infect Chemother 23 (2017) 45–50

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original Article

Retrospective survey of severe fever with thrombocytopenia syndrome in patients with suspected rickettsiosis in Japan

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ARTICLE INFO

Article history:
Received 30 June 2016
Received in revised form
18 September 2016
Accepted 23 September 2016
Available online 26 October 2016

Keywords:
Severe fever with thrombocytopenia syndrome
Japanese spotted fever
Rickettsiosis
Tick-borne infectious diseases

ABSTRACT

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne infectious disease caused by the SFTS virus (SFTSV). The aim of this study was to clarify whether SFTS is potentially misdiagnosed as rickettsioses, including spotted fever, typhus fever, and scrub typhus, which are also tick-borne disease. A total of 464 serum samples collected from 222 patients with clinically suspected rickettsiosis between 1999 and 2012 were tested for antibodies against the SFTSV. Of the 464 serum samples, one was positive for antibodies against the virus in an enzyme-linked immunosorbent assay and indirect immunofluorescence assay. The patient of SFTSV antibody-positive sample (15 days after disease onset) was positive for SFTSV genome in the acute phase sample (3 days after disease onset) as determined via reverse transcription-quantitative polymerase chain reaction. This patient, who was a resident of the Yamaguchi prefecture in Western Japan, was in his 40s when he showed symptoms in 2011. As the result, 1 of 222 patients, who was clinically suspected of rickettsiosis, was retrospectively diagnosed with SFTS. In this case, both the C-reactive protein and white blood cell count levels were lower than the ranges of these parameters for patients diagnosed with rickettsiosis. Therefore, SFTS should be considered in the differential diagnosis for rickettsiosis in Japan.

1. Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is caused by the SFTS virus (SFTSV), which belongs to the genus *Phlebovirus* in the family *Bunyaviridae*. SFTS was initially discovered as a novel bunyavirus infection in China, during which the disease name was proposed as SFTS [1]. SFTS is also reported to be endemic to Japan [2].

SFTSV was detected in several species of ticks, including *Haemaphysalis longicornis, Haemaphysalis flava, Amblyomma testudinarium, Ixodes nipponensis* [3], and *Rhipicephalus microplus* [4].

Tick-borne diseases, including rickettsioses such as Japanese spotted fever (JSF) caused by *Rickettsia japonica*, and several other spotted fever group (SFG) rickettsia species (*Rickettsia heilongjiangensis* and *Rickettsia tamurae*), are also prevalent in Japan [5–7]. Furthermore, *R. japonica* was isolated from *H. longicornis*, *H. flava*, *Haemaphysalis formonensis*, *Haemaphysalis cornigera*, *Haemaphysalis hystricis*, *Ixodes ovatus* and *Dermacentor taiwanensis* [6,8], and *R. tamurae* was isolated in *A. testudinarium* [7]. Thus, some ticks are vectors for both SFTSV and pathogenic *Rickettsia* species.

Therefore, we hypothesized that SFTSV infections may be present among patients with clinically suspected rickettsiosis in Japan. In this study, we retrospectively surveyed patients with SFTS using serum samples that had been collected from clinically suspected rickettsioses cases at the Department of Virology I, National Institute of Infectious Disease (NIID).

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http://dx.doi.org/10.1016/j.jiac.2016.09.011
1341-321X/

2. Materials and methods

2.1. Serum samples

A total of 464 serum samples collected from 222 patients with clinically suspected rickettsiosis were used in this study (Table 1). These serum samples had been sent to the Department of Virology I, NIID for etiological diagnosis of rickettsiosis between 1999 and 2012, and had been stored at −80 °C. The ages of the patients ranged between 1 month and 90 years. A total of 23 men, 22 women, and 11 sex-unknown patients were previously diagnosed with rickettsiosis (SFG rickettsiosis, typhus rickettsiosis, or scrub typhus) by demonstration of a significant increase in antibody titers against the respective pathogen, using methods described previously [6]. A sentence as follows is written in the materials section: Microbial examination results and final diagnosis of most of the patients included were not known except for the patients diagnosed as having rickettsiosis or SFTS in this study. This study was approved by the Ethics Committee for Medical and Health Research involving Human Subjects of the NIID (no. 474, January 16, 2014).

2.2. Enzyme-linked immunosorbent assay (ELISA)

Antigens used in the ELISA for detection of antibodies against SFTSV were 1% Nonidet P-40 (NP-40) treated-lysates of Huh-7 cells (obtained from BIKEN, Osaka University) infected with SFTSV (YG1 strain [2]) at a multiplicity of infection of 0.1 per cell. The mock antigen was derived in the same manner as the SFTSV antigen preparation but the Huh-7 cells were not infected with the SFTSV. The ELISA was performed as described previously [9]. Briefly, SFTSV and mock antigen-coated 96-well plates (Nunc™ Microwell™ 96 well plate, Thermo Fisher Scientific, Inc.,) were incubated with the heat-inactivated sera of the enrolled patients or SFTSV-antibody positive control serum [9], which were diluted 4-fold from 1:100 to 1:6400, as reported previously [10,11]. After washing the plates with 0.05% Tween-20 in PBS, the plates were incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (1:1000 dilution; ZiMax™ Goat Anti-Human IgG (H + L) HRP Conjugate; Thermo Fisher Scientific, Inc.). The plates were washed and then incubated with 100 μl ABTS [2, 2′azinobis (3-ethylbenzthiazolinesulfonic acid)] solution/well (Roche Diagnostics). The optical density at 405 nm (OD_{405}) for each well was measured against a reference OD at 490 nm. The adjusted OD_{405} was calculated by subtracting the OD of the negative antigen-coated wells from that of the corresponding wells. The cut off value was set 0.3 at the 1:100 dilution of serum for screening in this study based on values of SFTSV and mock-antigen coated wells.

2.3. Indirect immunofluorescence assay (IFA) for detection of SFTSV IgG

Antigen for the IFA was prepared referred to a previous study [2]. 2-fold diluted serum samples (from 1:100 to 1:3200, with PBS) were spotted on 12-well slide glass. Fluorescein isothiocyanate-conjugated rabbit anti-human IgG polyclonal antibody (1:150; DakoCytomation) was used as a second antibody. The wells of the glass slides were mounted with PermaFluor (Thermo Fisher Scientific, Inc.) and observed for immunofluorescent signals using a fluorescent microscope (Axioskop 2 plus; Carl Zeiss).

Table 1
The gender and diagnostic status in terms of JSF among the patients, whose serum samples were tested for rickettsiosis.

		Gender			
		Male	Female	No information	Total
Rickettsiosis	Positive	23 (4)	22 (7)	11 (3)	56 (14)
	Negative	77 (14)	60 (14)	29 (10)	166 (38)
Total		100	82	40	222

Parentheses: Number of patients from whom only single serum sample was tested for rickettsiosis.

2.4. Neutralization assay

The neutralization test was carried out as described previously [2], with minor modifications. Briefly, the serum samples were heat-inactivated (56 °C for 30 min) and serially diluted with Dulbecco's modified Eagle's medium containing 2% fetal calf serum (DMEM-FCS2). Subsequently, a 150 μL aliquot of each dilution was mixed with the same volume of DMEM-FCS2 containing 80 focus forming units (FFUs) of SFTSV YG1 [2], and incubated at 37 °C for 1 h. Vero cell monolayer (from American Type Culture Collection) were then incubated with each of the mixtures for 1 h at 37 °C, after which the Vero cells were overlaid with DMEM-FCS2 containing 1% methylcellulose. After a 6-day incubation period, the cells were fixed with formalin and stained with anti-SFTSV rabbit serum for 1 h [2], followed by incubation with HRP-conjugated goat anti-rabbit IgG (H + L) (Thermo Fisher Scientific, Inc.) for 1 h at 37 °C. FFUs for infected cells were detected using a peroxidase staining 3, 3-diaminobenzidine kit (Nacalai Tesque, Inc.). The number of FFUs was counted, and the neutralization titer was expressed as the reciprocal of the highest dilution level, at which FFUs were <50% of the control.

2.5. Amplification of S segment of SFTSV and phylogenetic analysis

Total RNA was extracted from 200 μL serum samples using a High Pure Viral RNA Kit (Roche Applied Science), according to the manufacturer's protocol. Complementary DNA was synthesized from the extracted RNA solution using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The SFTSV genome was amplified via polymerase chain reaction (PCR) and the nucleotide sequence of the PCR product, corresponding to the SFTSV nucleoprotein gene (168-628nt; size of PCR product, 461bp), was determined as previously described [12].

The nucleotide sequence of the partial S segment obtained in the study was analyzed using the Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and compared with those of other isolates registered in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Using these sequences, phylogenetic trees were constructed via the neighbor-joining method using MEGA6 software [13]. The accession numbers of the nucleotide sequences used for the phylogenetic analysis are shown in Table 2.

2.6. Analysis of clinical manifestations and laboratory data of patients

The clinical manifestations and laboratory data, including C-reactive protein (CRP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), platelet count (PLT), and white blood cell count (WBC), of patients with SFTS and those with rickettsioses (11 spotted fever rickettsiosis, 1 typhus rickettsiosis, and 4 scrub typhus) were compared. Data of SFTS were those of patients reported previously [2], and data of rickettsiosis were those of laboratory confirmed patients as having rickettsiosis among the 56 patients in the present study, for whom the data were available. The results of the comparison are presented as box and dot plots. A paired t-test was used for statistical analysis.