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Serological survey of severe fever with thrombocytopenia syndrome virus infection in Sika deer and rodents in Japan

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Abstract

Severe fever with thrombocytopenia syndrome (SFTS) is a newly recognized zoonosis that occurs in China, Japan, and South Korea and is caused by the SFTS virus (SFTSV), which is in the genus *Phlebovirus*, family *Phenuiviridae*. Since its discovery in Japan in 2013, SFTS has been reported in the western parts of the country. To elucidate the distribution of SFTSV, we conducted a serological survey of deer and rodents. Serum was screened using enzyme-linked immunosorbent assay (ELISA) and suspected cases were further tested with an indirect immunofluorescence antibody (IFA) assay. Serum samples from 315 deer from Hokkaido (non-endemic area), 41 deer from Miyazaki (endemic area), and 910 rodents from six locations in Japan were tested. Of the 41 deer from Miyazaki, 2 (4.9%) had high ELISA optical density (OD) values ($0.1 < OD < 0.3$) and a positive IFA result. All of the deer samples from Hokkaido were negative by ELISA ($OD < 0.1$). No SFTSV-positive rodents were found. Our results indicate that deer in Miyazaki were exposed to SFTSV, unlike deer from Hokkaido ($P < 0.05$).

Key Words: SFTS, Phlebovirus, Epidemiology, Deer, Rodents

Introduction

Severe fever with thrombocytopenia syndrome (SFTS) recently emerged as a public health threat in China,[15,16] Japan,[13] and South Korea[4] and has a case-fatality rate of 2 to 15%.[13,17] SFTS

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is caused by the SFTS virus (SFTSV), which belongs to the family *Phenuiviridae*, and was first isolated in China in 2009.[16] The disease caused by SFTSV is characterized by hemorrhagic fever with diarrhea, vomiting, drastic losses of leukocytes and platelets, disseminated intravascular coagulopathy, and multiorgan failure, which is the usual cause of death.[16] In Japan, SFTSV was first identified in 2013 and was traced back to as early as 2005 in a retrospective study of serum from patients who had had symptoms similar to those listed above.[13] Following the discovery of SFTSV in Japan, the virus was found to be endemic to western Japan, where SFTS patients continue to be reported annually.[13,19] SFTSV is transmitted by the ticks *Haemaphysalis longicornis*[8,14] and *Rhipicephalus microplus*, from which the viral RNA was detected in China and South Korea.[18] In Japan, the ticks *H. longicornis* and *Amblyomma testudinarium* were identified as SFTSV vectors,[20] although other tick species may also carry the virus. The recent discoveries of pathogenic tick-borne phleboviruses[9], like Heartland virus in the United States of America[10] and SFTSV in East Asia[1,16], have raised serious public health concerns. Elucidating the precise distribution of the pathogen within the regions and countries where patients are reported will provide useful information for disease risk assessment. By conducting serological surveys to estimate the prevalence of SFTSV antibodies in reservoir animals, we can determine the distribution of SFTSV in Japan. Serological studies of wild boar, deer, hedgehogs, wild rodents, hunting dogs, goats, sheep, cattle, chickens, and pigs suggest that a variety of animal species are infected with SFTSV.[2,7,12,14] In Japan, the SFTSV tick–vertebrate–tick cycle seems to be well established, as observed by the high SFTSV seroprevalence in deer, wild boar, dogs, and raccoon dogs in the areas where SFTS occurs.[19] Therefore, wild animals are useful sentinels for the sero-surveillance of SFTSV in Japan. Consequently, this study determined the geographic distribution of SFTSV activity in Japan. The data collected from this study will be useful for determining the risk of SFTSV transmission in endemic and non-endemic areas. Deer from Miyazaki and Hokkaido and rodents from six areas in Japan were screened using enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescent antibody (IFA) assay for the presence of anti-SFTSV antibodies in serum. By comparing the sero-prevalence in deer from Miyazaki with deer from Hokkaido, we have shown that the distribution of SFTSV activity was confined to the western parts of Japan in our sampling period, since antibody-positive animals were detected only in western Japan.

Materials and Methods

Study animals and sample collection: The sero-survey targeted animals in SFTS endemic areas in western Japan and non-endemic areas in northern Japan. A total of 356 sika deer (*Cervus nippon*) including 315 individuals of the subspecies of sika deer inhabiting Hokkaido (*Cervus nippon yesoensis*) and 910 rodents from Miyazaki, Toyama, Shimane, Gifu, Osaka, and Yamagata Prefectures in western Japan were screened (Fig. 1). The serum samples from the sika deer used in this survey were obtained by hunting and nuisance control culling from 2013 to 2014. The deer samples comprised 41 samples from Miyazaki Prefecture in western Japan and 315 from Hokkaido in northern Japan. The sample sizes and the inclusion of seven prefectures in this study were determined by the availability of samples. Banked rodent serum samples collected between 1997 and 2009 were included in our survey to assess whether SFTSV exposure could be traced back to before the virus was first identified. The methods of rodent capture and serum preparation are described elsewhere.[3]

Fig. 1. Map showing the survey sites in Japan and animal species collected from each site. Rodents were collected from six prefectures (circles), while deer were collected from two sampling sites in northern and western Japan (triangles). The grey areas are the prefectures where sika deer and rodents were captured.

Antigen preparation for the detection of anti-SFTSV antibodies: Hepatocellular carcinoma (Huh7) cells grown in a 75-mL flask were infected with SFTSV YG-1 strain (an isolate from an SFTS patient in Yamaguchi Prefecture, Japan[13]) at a multiplicity of infection of 0.1 and cultured for 3 days in Dulbecco's modified Eagle's minimum essential medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS). The cells were rinsed four times with phosphate-buffered saline (PBS). The cells were lysed with 1 mL of 1% NP-40 in PBS and incubated at room temperature for 10 min. The cell lysate was irradiated with ultraviolet (UV) light on a UV transilluminator for 10 min. The lysate was centrifuged at 12,000 rpm for 5 min in a microcentrifuge. The supernatant was collected and stored at −80°C until use. The lysate of mock-infected cells was prepared similarly.

Enzyme linked immunosorbent assay (ELISA): The serum samples were analyzed using an ELISA developed by the National Institute for Infectious Diseases in Japan. The SFTSV-infected Huh7 cell lysate was used as the antigen for the ELISA. First, 96-well EIA/RIA plates (Corning) were coated with 50 μL of SFTSV-infected Huh7 cell lysate antigen diluted to 1 : 800 in PBS and incubated overnight at 4°C. Mock-infected Huh7 cells were used as the control antigen and diluted similarly before applying to the plates. After incubation, the wells were washed four times with washing buffer composed of PBS and 0.05% Tween 20 (PBST). This was followed by blocking with 3% bovine serum albumin (BSA) and incubation at 37°C for 1 h. The washing was repeated as above. Control and test sera diluted in 3% BSA-PBST were applied to the plates and incubated at 37°C for 1 h. After washing, the plates were coated with horseradish peroxidase-conjugated chimeric protein A/G (Thermo Fisher Scientific) diluted in 3% BSA-PBST and incubated for 1 h at 37°C. Washing was repeated and 100 μl/well of *o*-phenylenediamine dihydrochloride substrate (Wako) was used for detection. The plates were incubated at room temperature for 15 min and the absorbance was measured using a spectrophotometer at 450 nm. The results are expressed as the difference between the optical density (OD) value of the control antigen and that of the SFTSV antigen. Samples were considered positive if the OD value was $>$ 0.1.

Indirect immunofluorescent antibody assay (IFA): Vero E6 cells grown in a 75-cm^2 flask were infected with SFTSV YG-1 strain and cultured for 3 days in MEM supplemented with 10% FBS. The cells were trypsinized, dispersed, and collected in a tube. The cells were seeded onto 24-well slides and cultured for 5 h in a CO_2 incubator. The slides were washed with PBS, fixed with acetone for 20 min, and used as IFA antigen slides. The fixed slides were stored at −80°C until use. Acetone-fixed SFTSV infected and non-infected (control) Vero E6 cells were incubated with serum diluted in PBS for 1 h, washed three times in PBS for 5 min each, and stained with Alexa Fluor 488 protein G (Thermo Fisher Scientific).

Finally, the slides were observed under a fluorescence microscope. A positive reaction was indicated by granular fluorescence in the cytoplasm of the Vero E6 cells and an antibody titer of $>$ 1/16. The antibody titer was determined as the reciprocal of the highest serum dilution that produced granular fluorescence in the cytoplasm.

Focus reduction neutralization test (FRNT): Two-fold serial dilutions of serum were prepared in 96-well plates with an initial dilution of 1/10. The SFTSV was then diluted in MEM supplemented with 2% fetal calf serum, penicillin G, and streptomycin. The virus was diluted in MEM to a concentration of 100 focus-forming units per 50-μL volume. Next, 50 μL of virus was added to each well and the virus-serum mixture was incubated for 1 h at 37°C in a CO_2 incubator. The inoculum was then applied to the Vero E6 cells and incubated for 1 h. The inoculum was removed and the cells were incubated for 3 days with an overlay of 1.5% carboxymethyl cellulose. To count the foci formed by each serum dilution, the plates were analyzed with the IFA assay, as described above. The neutralizing titer was determined as the reciprocal of the highest serum dilution that reduced the viral foci counts by 50% when compared with the negative control serum. Samples were considered positive if they tested positive on all three tests, *i.e.*, ELISA, IFA, and FRNT.

Statistical analysis: A one-tailed Fisher's exact test was used to compare the antibody prevalence rate between deer in Hokkaido (proportions of positive and negative sera) and deer in Miyazaki. A p-value $<$ 0.05 indicated statistical significance.

Results

Deer survey

This study surveyed 356 sika deer. Of the 41 deer from Miyazaki, 2 (4.9%) were sero-positive for SFTSV antibodies (Table 1). The ELISA OD values for the positive sera ranged from 0.1 to 0.3 (Fig. 2A). In the IFA, scattered cytoplasmic fluorescence was observed in SFTSV-infected Vero E6 cells (Fig. 3A and B) that were reacted with serum #268 and #326 respectively, but not with control serum (Fig. 3C). The two sera showed

Table 1. Sika deer screened for antibodies against SFTSV in northern and western Japan

Prefecture	Collection Year	Number screened	Confirmed positive, no. (%)
Hokkaido	2013	315	0 (0)
Miyazaki	2015	41	2 (4.9)*
Total		356	2 (0.6)

*$P < 0.05$

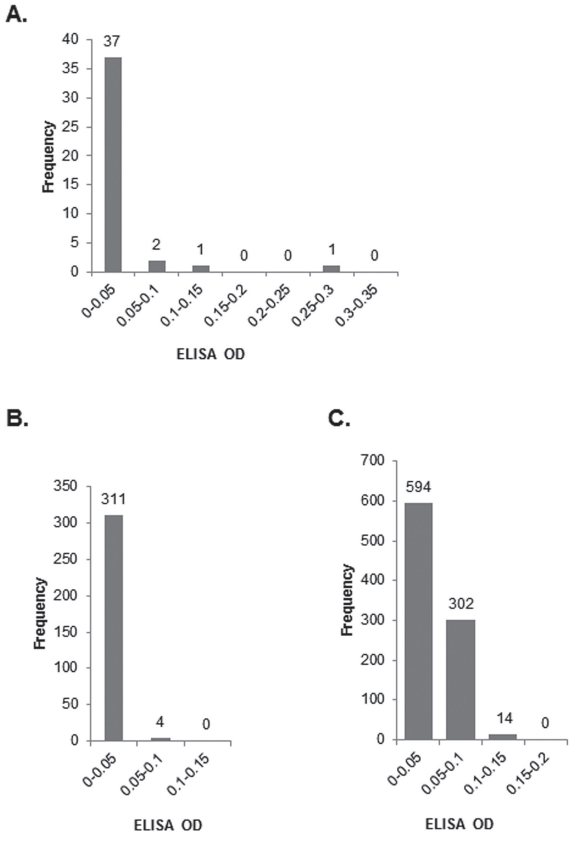

Fig. 2. Detection of severe fever with thrombocytopenia syndrome (SFTS) phlebovirus (SFTSV) antibodies by enzyme linked immunosorbent assay (ELISA). The frequency distribution of ELISA optical density values for deer from Miyazaki (A) and Hokkaido (B) and rodent serum (C).

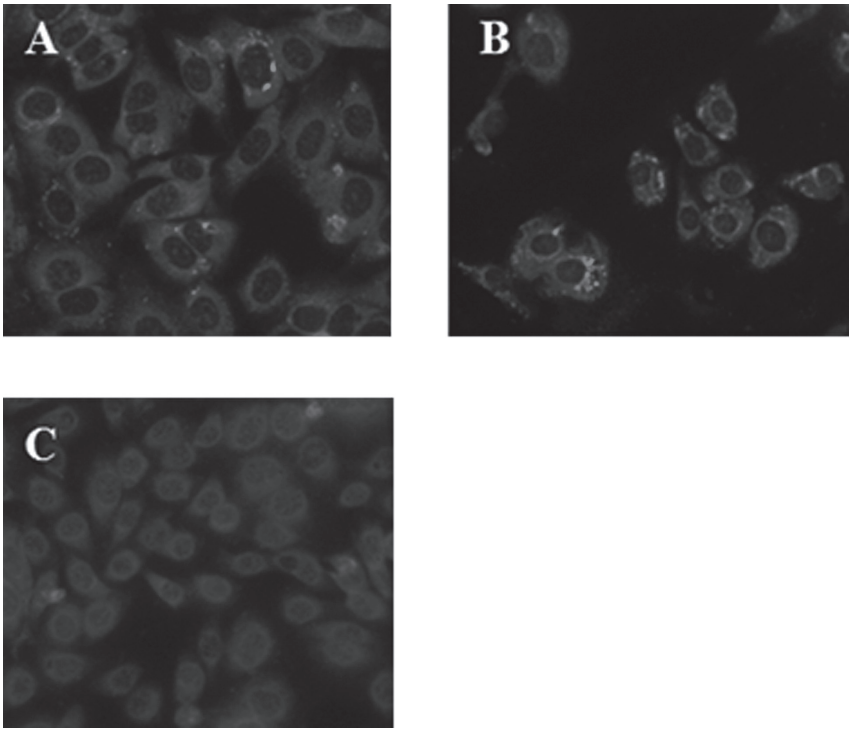

Fig. 3. Detection of SFTSV antibodies with the immunofluorescence assay. A and B; SFTSV-infected Vero E6 cells that reacted with deer serum (#268 and #362) showed cytoplasmic fluorescence in infected cells. (C) There was no fluorescence when the cells were stained with control serum.

IFA titers > 16 (#268: titer $= 64$ and #326; titer $= 128$). OD values ranging between 0 and < 0.1 were obtained by ELISA for the serum from Hokkaido deer, with the majority lying between 0 and 0.05 (Fig. 2B). When compared with deer from Miyazaki, the OD values for Hokkaido deer were significantly lower ($P = 0.02$). The $FRNT_{50}$ detected SFTSV neutralizing antibodies in deer serum from Miyazaki (Table 2), but not serum from Hokkaido (Hokkaido data not shown).

Table 2. Reactivity of deer serum on ELISA, IFA and focus reduction neutralization test

Sample Number	ELISA	IFA titer	50% focus reduction
268	+	64	40
326	+	128	320

Rodent survey

A total of 910 rodent serum samples were screened for antibodies against SFTSV (Table 3). These included 582 *Apodemus speciosus*, 51 *Apodemus argenteus*, 188 *Rattus norvegicus*, 1 *Rattus rattus*, 30 *Eothenomys smithii*, 19 *Eothenomys andersoni*, and 33 *Microtus montebelli*. The ELISA OD values ranged from 0 to 0.2, with the majority lying between 0 and 0.1 (Fig. 2C). Of the 910 rodent samples, 14 samples with OD values ≥ 0.1 were then tested by IFA and all 14 samples tested negative for anti-SFTSV antibodies. Serum was considered positive only if it tested positive by ELISA and IFA. Therefore, we concluded that all of the rodent serum samples were negative for anti-SFTSV antibodies. We sometimes encounter false positive samples by ELISA in sera of wild animals, especially in rodents. On the other hand, ELISA has an advantage to test a large number of samples. Therefore, we adopted the two-step strategy to detect anti-SFTSV antibodies in wild animals, ELISA for screening then IFA or FRNT for confirmation. Hayasaka *et al*. detected anti-SFTSV antibodies in wild boars by ELISA and

Table 3. Rodents screened for antibodies against SFTSV

Prefecture	Collection Year	Number screened	Positive, no. (%)
Shimane	1997–2009	118	0 (0)
Toyama	2000 & 2005	437	0 (0)
Yamagata	2008	65	0 (0)
Gifu	1997	176	0 (0)
Osaka	2000	28	0 (0)
Miyazaki	2001	86	0 (0)
Total		910	0 (0)

FRNT in a similar way[2].

Discussion

Using a combination of serological methods, we examined the prevalence of anti-SFTSV antibodies in deer from Miyazaki in western Japan and Hokkaido in northern Japan. We found that some deer in Miyazaki had anti-SFTSV antibodies, whereas deer from Hokkaido had no antibodies to SFTSV, implying that SFTSV infections in deer continue to occur in western Japan. SFTSV is reported to be prevalent in both wild and domestic animals.[12–14] In Japan, wild animals such as deer[19] and wild boar[2] have tested positive for antibodies to SFTSV and serve as sentinels for estimating the seroprevalence of anti-SFTSV antibodies, as well as for determining the geographic distribution of SFTSV. Seroprevalence studies in wild animals show that the antibodies are present in about 43.2% of the sika deer that were tested. In comparison, the percentage of seropositive wild boar ranges from 1.4 to 51%.[2,19] Kurihara *et al*. reported that the risk of SFTSV infection in Nagasaki was low due to the absence of seropositive humans, even among populations that were considered to be at a high risk of SFTSV infection.[5] Combined, these reports show that SFTSV infections in wild animals and humans are not widespread, even in areas where SFTSV is endemic. This concurs with our survey, which found a low SFTSV prevalence of 4.9% in deer from Miyazaki, a known SFTS endemic area. We extended the serological survey to Hokkaido, an area that is not endemic to SFTS, to assess the possible geographical expansion of SFTSV activity. Comparing the SFTSV seroprevalence in deer from Miyazaki and Hokkaido, we showed that SFTSV infections in deer have not spread to or are quite limited in Hokkaido. This concurs with the current status of SFTS patients being reported only in western Japan.[19]

In this survey, only serum samples were available, so we were limited to serological testing. With this limitation, a combination of serological tests is useful for screening for SFTSV activity. Interestingly, neutralizing antibodies were detected only in deer from Miyazaki and not deer from Hokkaido. Therefore, we concluded that deer from Miyazaki had been exposed to SFTSV or a closely related virus. A report on SFTSV seroprevalence in Nagasaki also found anti-SFTSV neutralizing antibodies in wild boar serum and the presence of a closely related virus was suggested.[2] The cross reactivity of SFTSV and Heartland virus has been reported in animal serum in the USA.[11] Going forward, it will be necessary to collect tissue samples from animals for virus isolation and detection of the SFTSV genome.

The role of rodents in the transmission of SFTSV is not clear. Seropositive rodents have been reported in China and viral RNA detected in rodent species,[6,7,12] suggesting that rodents are reservoir hosts for SFTSV. In our survey, no rodent serum samples collected between 1997

and 2009 in Japan showed any evidence of SFTSV antibodies. At this point, it is unclear whether the SFTSV seroprevalence was generally low during this sampling period in the regions that we surveyed or the rodents were captured from areas of low endemicity. Further serological surveys of rodents in Japan will be useful for monitoring the expansion of SFTS endemic areas and assessing the current risk for SFTSV infection in Japan. The results obtained in this survey will be useful for assessing the risk of SFTSV infection in Japan.

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Conflicts of interest

None to declare.

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