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Original Papers

Epidemiological Survey of Severe Fever with Thrombocytopenia Syndrome Virus in Ticks in Nagasaki, Japan

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Abstract: Severe fever with thrombocytopenia syndrome (SFTS) is an emerging disease endemic in East Asia. Transmitted to other organisms by infected ticks, the SFTS virus (SFTSV) and is endemic to Nagasaki in western Japan. However, epidemiological information regarding SFTSV in Nagasaki ticks has not been available to date. In this study, we began by examining the sensitivities of SFTSV gene detection by real-time RT-PCR and virus isolation in cultured cells and mice. These methods could detect SFTSV in the samples containing more than 4×10^0 ffu. Next, we attempted to isolate SFTSV and to detect viral gene in 2,222 nymph and adult ticks collected from May to August 2013 among seven regions of Nagasaki. However, neither virus isolation nor viral gene detection were confirmed in the tick pools. SFTSV positivity rates are considered to be very low in ticks, and viral loads are also very limited. Further investigations increasing the number of ticks and including larval samples as well as improved detection methods, may be required to find SFTSV-positive ticks in this region.
Key words: SFTSV, ticks, virus isolation, viral gene detection, real-time RT-PCR

Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging disease endemic in East Asia regions including China, Korea and Japan [1–4]. The clinical manifestations of SFTS include fever, diarrhea, organ dysfunction, thrombocytopenia and leukopenia, with fatality rates ranging from a few percent up to 30% [1–3].

The causative agent, the SFTS virus (SFTSV), belongs to the genus *Phlebovirus* in the family *Buniyaviridae* [3]. The SFTSV has been detected in ticks such as *Haemaphysalis longicornis* [5]. Furthermore, anti-SFTSV antibodies have been identified in animals including sheep, cattle and dogs [6–8]. Thus, the SFTSV naturally circulates between ticks and animals, and humans appear to be infected by the bite of a tick carrying the virus.

More than 100 cases of SFTS were identified in western Japan by 2014. Three quarters of the SFTS cases were reported from April to September (Yamaguchi Prefectural Surveillance Center of Infectious Disease, 2015), suggesting that the peak of incidence coincides with the tick density. In Nagasaki on the Japanese island of Kyushu, seven cases were identified from 2005 to 2014, and the patient in 2005 is the oldest among all SFTS cases reported worldwide [2]. The SFTS cases were distributed throughout the Nagasaki area. Thus, epidemiological surveys to determine tick species, infection rates and endemic areas are an important way to provide useful information and to promote disease prevention in this region.

In this study, we began by establishing SFTSV detection methods for virus isolation in cultured cells and mice as well as for viral gene detection by real-time RT-PCR, and then we collected ticks in several places and attempted to detect SFTSV.

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Methods

Virus and cells

The NagH2013-1 strain of SFTSV was isolated from an SFTS patient in Nagasaki in 2013. Stock virus of SFTSV was prepared from cell culture medium of Vero E6 cells and serially diluted in EMEM containing 2% FBS. Vero E6 cells were maintained in Eagle's Minimal Essential Medium (EMEM; Nissui Pharmaceutical Co.) containing 10% fetal bovine serum (FBS). All experiments using live SFTSV were performed in a biosafety level 3 laboratory at Nagasaki University according to standard BSL3 guidelines.

Focus forming assay

The SFTSV titer was determined using a focus forming assay. Confluent Vero E6 cells were inoculated with serially diluted culture supernatants of SFTSV and incubated in 2% FCS EMEM containing 1% methyl cellulose 4,000 (Wako Pure Chemical Industries, Ltd.) for five days. Viral foci were detected using SFTSV antiserum obtained from a recovered SFTS human case, peroxidase-conjugated anti-human IgG (American Qualex) and DAB substrate (Wako Pure Chemical Industries, Ltd.). Viral titers were expressed as ffu/ml. The experiment using human serum was performed with the approval of the ethics committee of the Institute of Tropical Medicine, Nagasaki University (approval number: 140829129).

Real-time RT-PCR

Viral copy numbers were examined by real-time RT-PCR. SFTSV-specific primers and a probe were designed based on the RdRp region of the consensus sequences of the L segment. The forward primer was SFTS_QPCR_965F: 5'-GCRAGGAGCAACAARCAAACATC-3', the reverse primer was SFTS_QPCR_1069R: 5'-GCCTGAG TCGGTCTTGATGTC-3' and the PrimeTime® qPCR probe was FAM/5'-CTCCCRCCC-3'/ZEN/5'-TGGCTACCAAA GC-3'/IBFQ (Integrated DNA Technologies). The RT-PCR reaction was performed using a One Step PrimeScript RT-PCR Kit (Takara Bio Inc.) and a 7500 Real-time RT-PCR System (Applied Biosystems). The copy numbers were calculated as a ratio of the copy numbers to the standard control. Standard SFTSV RNA was prepared from a cloned plasmid vector, which contained a previously described RdRp insert generated by RT-PCR [9].

Virus isolation in Vero E6 cells

The stock virus was serially diluted ten-fold in S-1 (4×10^5 ffu/ml) to S-7 (4×10^{-1} ffu/ml). Ten microliters of each SFTSV S-1 to S-7 samples was inoculated into confluent Vero E6 cells cultured in 12- or 24-well plates, in 1 ml or 0.5 ml of 2% FBS EMEM, respectively. After seven days, 100 ul of supernatant was transferred to fresh Vero E6 cells in the same plates. After an additional seven days, supernatant was harvested and RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen). Virus detection was confirmed by real-time RT-PCR. Our preliminary experiments showed that extracted samples from ticks did not significantly inhibit the reactions.

Virus isolation in mice

A129 mice were purchased from B & K Universal Limited and bred in the animal facility at Nagasaki University. Ten microliters of each SFTSV S-1 to S-7 sample was intracerebrally inoculated into 1- to 2-day-old suckling mice. The mice were observed for clinical signs daily for seven days. The brains were collected from dead mice or survivors at day 7 because our previous and preliminary data showed that virus infection and disease development were observed within seven days.

RNA was extracted from brains using the RNeasy Lipid Tissue Mini Kit (Qiagen). Virus detection was confirmed by real-time RT-PCR. The animal experiments were performed in accordance with the recommendations found in the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions, under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The experimental protocols were approved by the Animal Care and Use Committee of Nagasaki University (approval number: 1401201115, 1408051166).

Tick collection and SFTSV detection

Ticks were collected by flagging in seven places throughout Nagasaki from May to August 2013 (Fig. 1). Some ticks were collected from captured deer. The collected ticks were grouped according to species, stage (nymph or adult), sex and area of collection, and 1 to 30 ticks were placed together in each of the pools.

Pooled ticks were homogenized using the Micro Smash™ MS-100R (TOMY DIGITAL BIOLOGY CO., LTD) with one stainless bead (4.8 Ø) and 0.5 or 1 ml of 2% FBS EMEM per reaction tube at 4,500 rpm for 30 seconds at 4°C. One-hundred microliters of homogenized samples were inoculated into cultured Vero E6 cells. After seven days, 100 ul of supernatant was transferred to fresh Vero E6 cells. After an additional seven days, supernatant was harvested and RNA was extracted. RNA was extracted from 250 ul of homogenized tick samples using Isogen-LS (Nippon gene). Virus detection was confirmed by real-time RT-PCR.

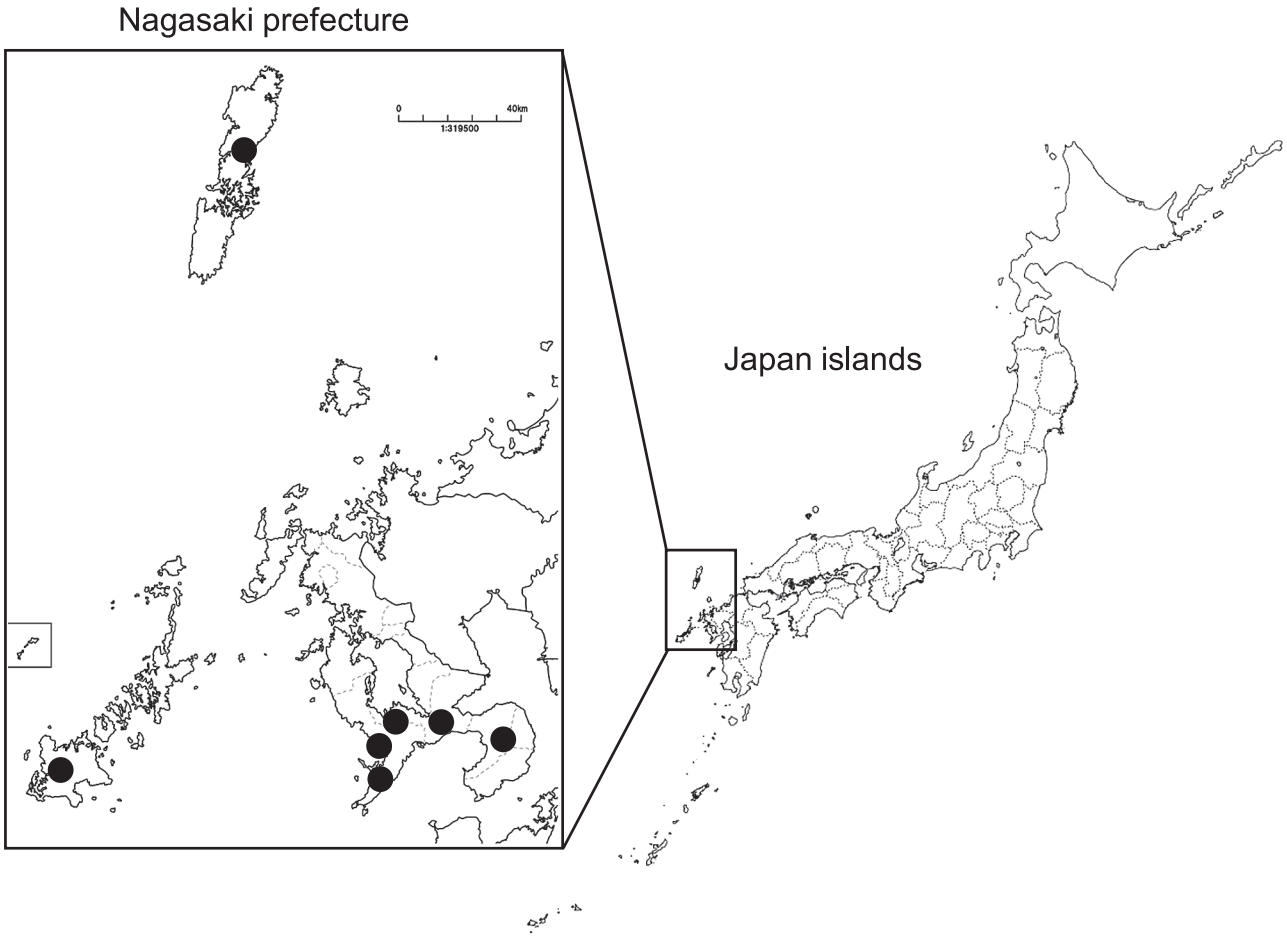

Fig. 1. Map showing Nagasaki Prefecture in the Kyushu islands, Japan. Black circles indicate the places where ticks were collected.

Results

Sensitivity of virus isolation using Vero E6 cells and A129 mice

To examine the sensitivity of virus isolation, 10 ul of the S-1 to S-7 samples equivalent to 4×10^{3} to 4×10^{-3} ffu was inoculated into Vero E6 cells and then passaged into fresh Vero E6 cells. Virus propagation was confirmed in the culture fluids. SFTSV was detected in the S-1 to S-4 inoculated cells in both the 12- and 24-well plates, indicating that this procedure can isolate SFTSV in samples containing more than 4×10^{0} ffu of the virus (Table 1).

We also investigated the sensitivity of virus isolation in A129 suckling mice by intracerebral inoculation with 10 ul of the S-1 to S-7 samples. Virus propagation was confirmed in the brains. Here, we performed intracerebral inoculation in suckling mice because they generally appeared to be more susceptible than adult mice and because the intracerebral route was the easiest way to conduct inoculations. Mice inoculated with S-1 to S-4 died after three to five days, although it was difficult to observe the clinical signs. SFTSV was detected in the brains of dead mice (Table 1). No virus was detected in the brains of mice inoculated with the S-5 to S-7 samples, indicating that the inoculation in A129 mice could detect SFTSV in samples containing more than 4×10^{0} ffu of virus (Table 1).

These results suggested that the sensitivities of virus isolation using Vero E6 cells and A129 mice were equivalent and that both methods could isolate the virus from samples containing more than 4×10^{0} ffu of SFTSV. Thus, we used Vero E6 cells to isolate SFTSV for further experiments.

Sensitivity of viral RNA detection by real-time RT-PCR

To determine the sensitivity of real-time RT-PCR specific to the SFTSV RdRp gene, detectable copy numbers were examined for RNA transcribed from the cloned

Table 1. Sensitivity of SFTSV isolation methods and viral RNA detection

Sample	S-1	S-2	S-3	S-4	S-5	S-6	S-7
ffu[a]	4×10^3	4×10^2	4×10^1	4×10^0	4×10^{-1}	4×10^{-2}	4×10^{-3}
Virus isolation*							
Vero E6 (12)[b]	+ + +	+ + +	+ + +	+ + +	+ − −	+ − −	− − −
Vero E6 (24)[c]	+ + +	+ + +	+ + +	+ + +	+ − −	− − −	− − −
Virus isolation**							
Mice	+ +	+ +	+ +	+ +	− −	− −	− −
RNA detection**							
Real-time RT-PCR	+ +	+ +	+ +	+ +	− −	− −	− −

[a] titer of virus inoculum expressed as focus-forming units (ffu) present in 10 ul of S-1 to S-2
[b] 12-well plate
[c] 24-well plate
* done in triplicate
** done in duplicate
+: positive detection, each sign representing one well, one mouse or one sample
−: negative detection, each sign representing one well, one mouse or one sample

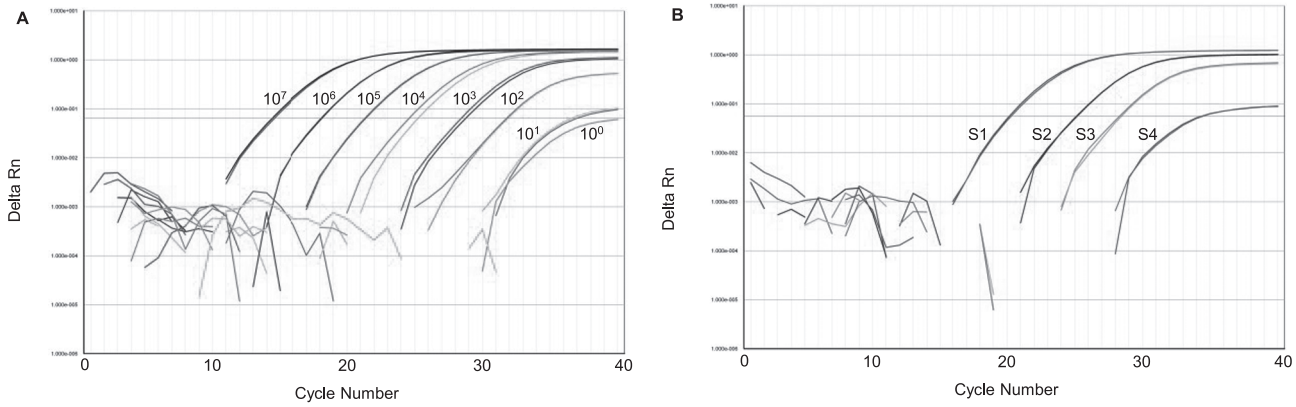

Fig. 2. Real-time kinetics of TaqMan real-time RT-PCR with SFTSV-specific primer/probe set. X-axis indicates the cycle numbers while Y-axis shows the delta Rn. Delta Rn is the normalization of Rn obtained by subtracting the baseline. Rn is the ratio of the fluorescence emission intensity of the reporter dye. (A) *In vitro* transcribed RNA of SFTSV was serially diluted. The numbers 10^0 to 10^7 indicate the copy numbers that were identified. (B) RNA was extracted from serially diluted S-1 to S-7 samples, and the limit of RNA detection in each sample was examined. S1 to S4 indicate the reaction samples of S-1 to S-4 that were determined to contain 4×10^3 to 4×10^0 ffu of SFTSV. The reactions were repeated three times and a representative result is shown.

cDNA of an SFTSV gene. The real-time RT-PCR could detect more than 10^0 copies of synthesized RNA (Fig. 2A).

We next examined the sensitivity of the real-time RT-PCR using RNA extracted from infectious viruses. RNA was extracted from 100 ul of S-1 to S-7 samples, and one of ten volumes of each extract (equivalent to 10 ul of sample) was used in each reaction. The real-time RT-PCR could detect viral RNA in the S-1 to S-4 samples (Fig. 2B), indicating that this method can detect SFTSV in samples containing more than 4×10^0 ffu of the virus (Table 1).

The results showed that there is no difference in sensitivity between virus isolation using cultured cells and mice and viral RNA detection by the real-time RT-PCR.

Collected ticks in Nagasaki

A total of 2,222 ticks of nymphs (74.5%) and adults (female 14.0%, male 11.5%) were collected in seven areas of Nagasaki and were examined for SFTSV detection (Table 2). The analyzed tick species were *H. longicornis* (76.9%), *H. formosensis* (15.2%), *H. megaspinosa* (2.3%), *H. flava* (1.0%), *H. hystris* (0.6%), *A. testudinarium* (1.7%) and *H. yeni* (2.3%). The collected ticks included four adult ticks of *H. longicornis* from deer. These ticks were grouped into 576 pools according to species and stage of life.

Table 2. Distribution of ticks according to species, life stage, sex and area of collection and the results of SFTSV detection based on pooled samples

Species	Stage Sex	Area of collection							Total	Positive pools	
		A	B	C	D	E	F	G		RNA	Virus
H. longicornis	nymph	525	344		26	17	308		1220	0	0
	female	16	20		9	1	226		272	0	0
	male	13	41		12		151		217	0	0
H. formosensis	nymph	253		7	28	4			292	0	0
	female	7	1		12	1			21	0	0
	male	10		1	13	2			26	0	0
H. megaspinosa	nymph	51							51	0	0
	male				1				1	0	0
H. flava	nymph	14	2						16	0	0
	female	2			1				3	0	0
	male	1			1	1			3	0	0
H. hystricis	nymph	1			1				2	0	0
	female		2	3	3	1			9	0	0
	male				2				2	0	0
A. testudinarium	nymph	20	1		6	6	4		37	0	0
H. yenii	female							31	31	0	0
	male							19	19	0	0
Total		913	411	11	115	33	689	50	2222	0	0

No virus detectable from collected ticks

We first tried to isolate SFTSV by inoculating all homogenized pooled ticks onto Vero E6 cells, however, no virus was detected from the culture fluid of these infected cells (Table 2). We also tried to detect viral genes in the extracted RNA from tick pools by real-time RT-PCR, but again, no positive samples were identified in any of the pools.

Discussion

In this study, we attempted to detect SFTSV in ticks collected in Nagasaki, Japan, where SFTS is endemic. We showed that virus isolation in Vero E6 cells and SFTSV specific real-time RT-PCR could detect SFTSV in samples containing more than 4×10^0 ffu of the virus, but when we tried to find SFTSV-positive ticks using these methods, no virus was detected.

Zhang et al. showed that SFTSV (Huaiyangshan virus) RNA was found in *H. longicornis* and *R. microplus* [5]. They examined more than 17,000 ticks and the minimum infection rate was approximately 0.13% (23 positive pools/17,731 ticks). Park et al. reported the prevalence of SFTSV in *H. longicornis* in South Korea [10]. They examined more than 10,000 ticks and the minimum infectious rate was 0.46%. On the other hand, Ham et al. showed that SFTSV genes were not detectable in approximately 800 ticks collected in Seoul [11]. In these studies, the authors performed real-time RT-PCR for the detection of SFTSV genes, but the isolation of infectious virus was not described. Although copy numbers of viral RNA were not mentioned in these studies, the Ct values of positive samples by this method was 38 cycles, and thus viral copy numbers appeared to be very low [10]. Therefore, SFTS-positive tick rates are very low and viral loads in the ticks are very limited.

Our real-time RT-PCR protocol could detect viral RNA in samples containing more than 4×10^0 ffu. The RNA concentration in S-1 (4×10^5 ffu/ml) was 2×10^7 copies of RNA/ml, and thus 10 ul of S-4 appeared to be equivalent to 2×10^2 copies of viral RNA. Therefore, although the real-time RT-PCR could detect 10^1 copies of synthesized RNA, at least 2×10^2 copies of RNA were required for detection from infectious SFTSV samples. Thus, if viral loads in the ticks are very limited (e.g., less than 10^2 copies of viral genes) our real-time RT-PCR protocol may not detect SFTSV in ticks. We have completed cDNA cloning of the N gene for protein expression by E. coli in our laboratory, and we targeted the RdRp gene of the L segment for real-time RT-PCR to avoid contamination of the N gene. Methodological improvements in real-time RT-PCR (e.g., targeting other sequences in the L or M genes)

may be effective when attempting to identify SFTSV-positive ticks by viral RNA detection.

In this study, we primarily tried to isolate infectious virus from ticks using Vero E6 cells because infectious virus isolation is necessary to ensure that ticks do indeed harbor and transmit SFTSV. Here, we examined nymph and adult ticks but not larva. Although there are limited data, larva may be a key to successful SFTSV isolation. Further investigations increasing the number of ticks, including larva, may be required to find SFTSV-positive ticks in this region.

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