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# Roles of raccoons in the transmission cycle of severe fever with thrombocytopenia syndrome virus

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**ABSTRACT.** The present study investigated severe fever with thrombocytopenia syndrome virus (SFTSV) infection in raccoons in Wakayama Prefecture from 2007 to 2019. To perform surveillance, an enzyme-linked immunosorbent assay (ELISA) was established, and the sensitivity and specificity of the ELISA were 100% in comparison with a 50% focus-reduction neutralization assay. Using the established ELISA, we performed serosurveillance of SFTSV infection in 2,299 raccoons in Tanabe region, Wakayama Prefecture from 2007 to 2019. The first anti-SFTSV-positive raccoon was captured in October 2009. The seroprevalence of SFTSV infection was <10% between April 2009 and March 2013, 23.9% between April 2013 and March 2014, 37.5% between April, 2014 and March 2015, and over 50% from April 2015. Next, we performed detection of SFTSV genes in sera of raccoons captured in Wakayama Prefecture after April 2013. The results indicated that 2.4% of raccoons were positive for SFTSV genes and that the frequency of SFTSV infection among raccoons between January and March (0.7%) was lower than that between April and June (3.4%). In addition, virus genes were detected from many specimens, including sera and feces of two raccoons, and viral antigens were detected in lymphoid cells in lymphoid follicles in the colon by immunohistochemical staining. In conclusion, SFTSV had recently invaded the area and had rapidly spread among wild animals. The first patient in this area was reported in June 2014, indicating that raccoons are good sentinels for assessing the risk of SFTSV in humans.

**KEYWORDS:** raccoon, severe fever with thrombocytopenia syndrome, wild animal

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Severe fever with thrombocytopenia syndrome virus (SFTSV) belongs to Order *Bunyavirales*, Family *Phenuiviridae*, Genus *Bandavirus* and is the agent that causes SFTS in humans. The first case of SFTS was reported in China, followed by cases in Japan, South Korea, Vietnam and Taiwan [13, 22, 24, 27, 30]. Since 2013, human cases of SFTS have been continuously reported in the western part of Japan and over 100 cases have been reported in 2019. The case fatality ratio is very high (27%) and the clinical symptoms include hemorrhagic fever similar to Crimean-Congo hemorrhagic fever [8, 10].

SFTS is known as a tick-borne zoonosis and to circulate between ticks and mammals, including domestic and wild animals [12, 17, 18, 28]. SFTSV can infect many mammalians and causes various clinical signs. In China, livestock animals, such as cattle, sheep and goats seem to be inapparently infected with SFTSV and act as amplifying hosts in pathogen transmission [3, 4, 17]. On the other hand, clinical signs due to SFTSV infection have also been reported in zoo and domestic animals in Japan [15, 16, 21]. Surveillance showed that both antibodies and viral RNA could be detected from several wild animals, including wild boar, deer, hedgehogs, and rodents [2, 3, 6, 9, 12]. In addition, it is suggested that migratory birds might carry SFTSV-infected ticks and contribute to the transboundary spreading of SFTSV [29, 31]. Although wild animals would play important roles in the transmission cycle of SFTSV, the roles of wild animals in nature have not been fully clarified.

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Serological surveillance of SFTSV infection indicates that rates of seropositivity for SFTSV are high in wild animals, such as wild boar and deer, in endemic areas [6, 9, 18]. Therefore, investigations of SFTSV infection among wild animals are important for indirectly assessing the risk of infection in humans and other animals and can be expected to contribute to clarification of the mechanism of SFTSV circulation in nature.

In the present study, we performed retrospective surveillance of SFTSV infection in raccoons using sera collected from raccoons in Wakayama Prefecture since 2007.

## MATERIALS AND METHODS

### *Cells and viruses*

Vero cells (Japanese Collection of Research Bioresources [JCRB] number: JCRB9013) derived from African green monkey were cultured in Dulbecco's modified Eagle's medium (DMEM: Thermo Fisher Scientific, Rockford, IL, USA) with 5% heat-inactivated fetal calf serum (FCS; Thermo Fisher Scientific), and 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific) at 37°C under 5% $CO_2$. HuH-7 (JCRB0403) cells derived from human hepatocellular carcinoma were maintained in DMEM with 5% FCS and 100 U/ml penicillin and 100 µg/ml streptomycin.

SFTSV strain HB29 was kindly provided by Drs. Xin Li and MiFang Liang of the Chinese Center for Disease Control and Prevention, Beijing, People's Republic of China [30]. The virus was propagated in Vero cells and stored at −80°C until use.

### *Serum and tissue samples*

Serum samples were collected from a total of 4,129 raccoons (*Procyon lotor*) between 2007 and 2019 in Wakayama Prefecture, Japan (Fig. 1). These animals were captured as countermeasures under the official population control program and sampling was approved by the local government. Most of blood and tissues were collected within 2 hr after death and the latest sampling was within 5 hr. Body weight was measured using spring scale. Age was classified into 3 age classes, 0, 1 and 2+, based on observation on canine pulp cavity, cranial suture, and femur epiphysis. All collected sera were stored at −20°C until use.

Tissue samples were collected from two raccoons (SRH186 and SRH187) on June 2014 for virus detection by RT-PCR, detection of viral antigens by immunohistopathology, and virus isolation.

### *Enzyme-linked immunosorbent assay (ELISA)*

To determine the specific antibody against SFTSV in animal sera, enzyme-linked immunosorbent assay (ELISA) was performed

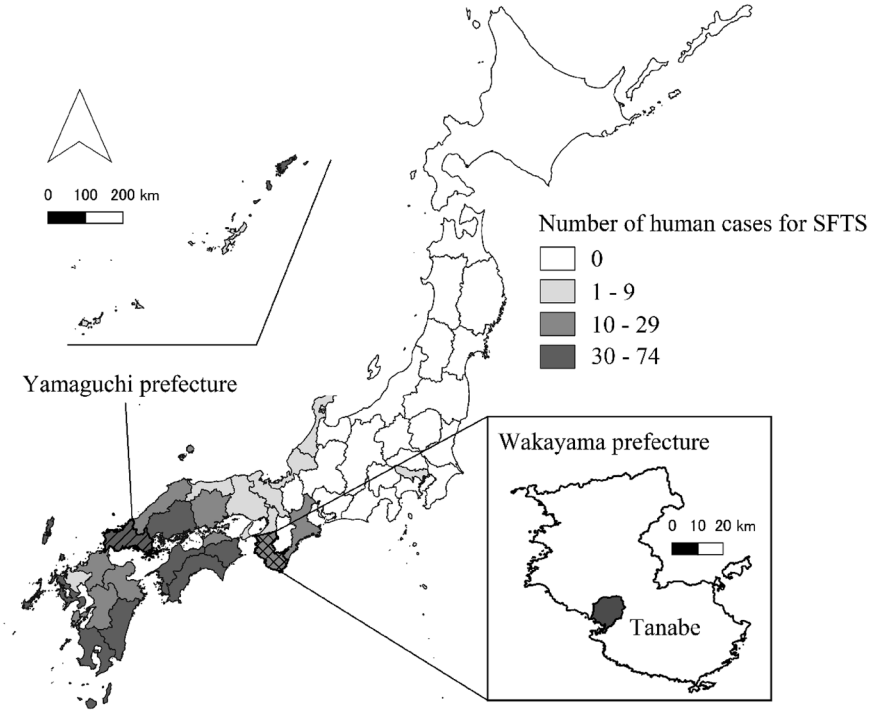


**Fig. 1.** Map of the severe fever with thrombocytopenia syndrome virus (SFTSV)-endemic areas in Japan. Graduation of white to black indicates the number of human cases in the prefecture until 2020 (National Institute of Infectious Diseases (NIID) homepage; https://www.niid.go.jp/niid/ja/sfts/3143-sfts.html). Diagonal and orthogonal lines indicate Yamaguchi and Wakayama Prefectures, respectively. Tanabe region, which is located in central Wakayama Prefecture, is shown in the magnified map of Wakayama Prefecture.

using extracts from SFTSV HB29- or mock-infected HuH-7 cells [5, 9]. In brief, antigens were coated with coating buffer (0.05 M carbonate-bicarbonate buffer, pH9.6) in an ELISA plate (MaxiSorp; NUNC, Roskilde, Denmark), incubated at 37°C for 2 hr, and blocked by 1% Block Ace (KAC, Kyoto, Japan) in PBS at 37°C for 30 min. As the 1st antibody, serum was diluted to 1:100 in 0.4% Block Ace in PBS containing 0.05% of tween 20 (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) (PBS-T) and 100 µl was added to duplicate wells. After incubation at 37°C for 30 min, Protein A/G conjugated with HRP (Thermo Fisher Scientific) diluted in 0.4% Block Ace in PBS-T was used as the secondary antibody. These reactions were visualized by ABTS Peroxidase Substrate (SeraCare Life Science, Milford, MA, USA) and the optical density (OD) was measured by a microplate reader (Bio-Rad, Hercules, CA, USA) using a 405 nm filter. For raccoons, an ELISA cut-off value of 0.564 was applied after comparison with a virus-neutralizing test.

### *Fifty percent focus-reduction neutralization test ($FRNT_{50}$)*

A virus-neutralizing (VN) test for SFTSV was conducted using a 50% *focus*-reduction neutralizing test ($FRNT_{50}$). Approximately 2,000 focus-forming units/ml of SFTSV HB29 strain were equally mixed with inactivated serum, which were diluted two-fold from 1:5 with DMEM containing 2% FCS and 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific) and incubated at 37°C for 1 hr. Then, 100 µl of the mixture was inoculated onto monolayered Vero cells in a 12-well plate (SUMITOMO BAKELITE, Tokyo, Japan). After 1 hr of adsorption, cells were washed with DMEM and overlaid with DMEM containing 2% FCS and 1% methylcellulose and cultured at 37°C in 5% $CO_2$ for seven days. Cells were fixed by 10% buffered formalin, exposed to UV irradiation and permeabilized by PBS containing 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). Infected cells were stained by rabbit antibodies against SFTSV N protein [26] and Protein A/G conjugated with HRP (Thermo Fisher Scientific) using 3,3′-diaminobenzidine tetrahydrochloride hydrate (DAB; FUJIFILM Wako Pure Chemical Corp.). Serum samples that reduced the number of focuses to ≤50% of the number in control wells were considered to be positive.

### *Receiver operating characteristic (ROC) analysis*

To determine the cut-off value of the ELISA for surveillance in raccoons, a receiver operating characteristic (ROC) analysis was performed between the ELISA and the $FRNT_{50}$ using the the EZR software program (version 3.5.3) [7]. The sensitivity and specificity were estimated by the $FRNT_{50}$ as the gold standard.

### *Virus isolation*

Tissue specimens were subjected to the preparation of homogenates in DMEM with 1% FCS, 50 µg/ml kanamycin, 50 µg/ml gentamycin, 100 U/ml penicillin and 100 µg/ml streptomycin. Then, the supernatants were harvested by centrifugation at 12,000 × *g* for 7 min. Sera, swabs, and tissue specimens were inoculated to Vero cells, and the cells were cultured until a cytopathic effect (CPE) was observed.

### *RNA extraction*

RNA was extracted from sera and supernatant of cell cultures using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). RNA was extracted from tissue samples using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions.

### *Reverse transcription-polymerase chain reaction (RT-PCR)*

One-step RT-PCR was performed using a One-step RT-PCR kit (QIAGEN) with two primers (S2-200: 5′-GACACAAAGTTCATCATTGTCTTTGCCCT-3′, S2-360: 5′-TGCTGCAGCACATGTCCAAGTGG-3′) [20]. PCR was performed as follows, 1 cycle of 50°C for 30 min and 95°C for 15 min, 40 cycles 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, and 1 cycle of 72°C for 7 min. PCR products was visualized by electrophoresis and positive PCR products were purified by MinElute Gel Extraction kit (QIAGEN). The sequence was determined using BigDye Terminator v.3.1 technology (FASMAC, Atsugi, Japan). The sequences were deposited into the DNA Data Bank of Japan (DDBJ; Accession numbers: LC579721-579749).

### *Real time RT-PCR*

The quantitative detection of SFTSV was performed by real time RT-PCR. RNA was extracted from tissue supernatants for virus isolation. The forward primer was SFTSV-S2-237s: 5′-GCA ACA AGA TCG TCA AGG CAT CAG G-3′, the reverse primer was SFTSV-S2-400a: 5′-TGC TGC AGC ACA TGT CCA AGT GG-3′ and the MGB probe was SFTSV-S2-317MGB: 5′-CTG GTT GAG AGG GCA-3′. RT-PCR was performed using a One Step PrimerScript RT-PCR Kit (Takara Bio, Kusatsu, Japan) and StepOne Real-Time RT-PCR System (Thermo Fisher Scientific), under the following conditions: 1 cycle of 42°C for 5 min and 95°C for 10 sec and 50 cycles of 95°C for 5 sec, and 64°C for 60 sec. The copy numbers were calculated using StepOne Software V2.1 (Thermo Fisher Scientific).

### *Analysis of whole genome sequence*

The whole genome sequences of SFTSV isolated from four raccoons (TNB1580, TNB1590, SRH186 and SRH187) were determined by next-generation sequencing (NGS) with MiSeq (Illumina, San Diego, CA, USA). Genomic DNA libraries for each of the strains were constructed using NEBNextUltra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) and NEBNext Multiplex Oligos for Illumina (Index Primer Set 1 or 2) according to the manufacturer's

instructions. The DNA libraries were analyzed by the MiSeq system (Illumina) using Illumina MiSeq Reagent Kit 300 cycle v2 (Illumina). Then, the reads were *de novo* assembled with the CLC Genomic Workbench 7.0 software program (CLC Bio/ Qiagen, Aarhus, Denmark). The whole genomes were deposited into DDBJ (Accession numbers: LC579709–579720).

### *Phylogenetic analysis*

The nucleotide sequences obtained from the four raccoons were aligned with 45 other sequences deposited in the DDBJ using Clustal W and a phylogenetic tree was constructed by the Maximum likelihood method based on the General Time Reversible model using the MEGA 7.0.26 software program [11]. The confidence of the tree was evaluated by 1,000 bootstrap replications. Guertu virus (NCBI accession number: NC_043609) was used as an outgroup to construct phylogenetic tree [1].

### *Histopathology and immunohistopathology*

Tissue specimens, including heart, lung, liver, spleen, stomach, small intestine, and colon were collected from two raccoons, SRH186 and SRH187, for histopathological examinations. The collected tissues were fixed in 10% buffered formalin, processed routinely into paraffin, sectioned, and stained with hematoxylin and eosin (H&E) and examined by microscopy. Immunohistochemical (IHC) staining was also performed to identify SFTSV antigens in tissues using a rabbit polyclonal antibody against SFTSV N protein [25].

### *Statistical analysis*

The $\chi^2$ test was performed to analyze the difference in seroprevalence. Fisher's exact test was used to evaluate small positive numbers in gene detection. For all tests, $P$ values of <0.05 were considered statistically significant.

## RESULTS

### *Establishment of the ELISA for the detection of anti-SFTSV antibodies in raccoons*

We determined the exact cut-off value for the further analysis of SFTSV infection in raccoons. For this purpose, serum samples from 111 raccoons collected in Wakayama Prefecture in 2018 were compared by $FRNT_{50}$ and ELISA. $FRNT_{50}$ showed that 81 raccoon sera (73%) were positive for SFTSV. Based on an ROC analysis, the cut-off value was set at 0.564, which resulted in 100% sensitivity and 100% specificity (Table 1). When this cut-off value was applied to the surveillance of SFTSV infections, 1,334 of 4,129 raccoons (32.3%) in Wakayama Prefecture were found to possess anti-SFTSV antibodies.

### *Seroprevalence of SFTSV infection in raccoons in Tanabe region, Wakayama Prefecture*

To perform a further epizootiological study, we analyzed 2,299 raccoon samples obtained from Tanabe region, a city in Wakayama Prefecture, because information on raccoons was recorded in detail. The seroprevalence of SFTSV infection among raccoons was compared by year (Fig. 2 and Table 2). The first SFTSV antibody-positive raccoon was detected in October 2009. Before March 2013, the seroprevalence was <10%. However, the seroprevalence had rapidly increased since April 2013, and a high seroprevalence rate of approximately 50% was maintained after April 2015 (Fig. 2 and Table 2).

As a further analysis, we focused on the endemic period from April 2015 to March 2019. A total of 1,461 raccoons were compared according to sex, age, and body weight (Table 3, Fig. 3). There was no significant difference between males and females. Interestingly, 83.2% of over raccoons that were >2 years of age possessed antibodies against SFTSV. Furthermore, 66.3% of raccoons with a body weight of <2 kg were positive, while only 30.0% of raccoons with a body weight of 3–4 kg were positive.

### *Detection of SFTSV genes from raccoons*

The detection of SFTSV RNA were performed from selected 1,374 raccoon sera during April 2013 and March 2019, revealing that 33 raccoons (2.4%) were positive (Tables 4 and 5). Although the prevalence of SFTSV RNA in raccoons captured in January, February, March, April and September was 1%, 1%, 0%, 1.7% and 0.9%, respectively, that in June (6%) was significantly higher (Table 4). From January to March the prevalence of SFTSV RNA in raccoons (0.7%) was significantly lower than that (3.4%) from April to June. However, there was no significant difference in the prevalence of the SFTSV genome according to sex, age, or body weight (Table 5).

**Table 1.** Comparison of the enzyme-linked immunosorbent assay (ELISA) and 50% focus-reduction neutralization test ($FRNT_{50}$) results

| | | ELISA absorbance | | Total |
|---|---|---|---|---|
| | | ≥0.564 | <0.564 | |
| $FRNT_{50}$ | Positive | 81 | 0 | 81 |
| | Negative | 0 | 30 | 30 |
| Total | | 81 | 30 | 111 |

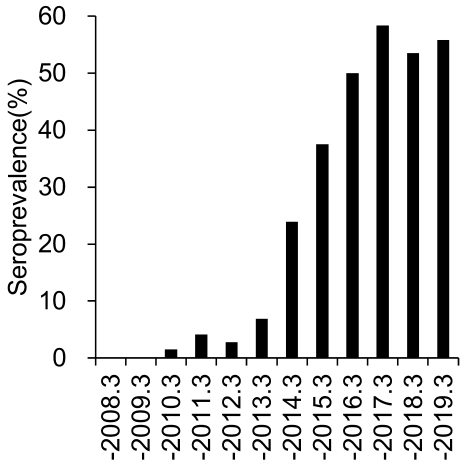


**Fig. 2.** Change in the seroprevalence of severe fever with thrombocytopenia syndrome virus (SFTSV) infection among raccoons in Tanabe region (n=2,299) by year.

**Table 2.** Prevalence of anti-severe fever with thrombocytopenia syndrome virus (SFTSV) antibodies among raccoons in Tanabe region

| Period of sample collection | Number of examined animals | Number of positive animals | Percentage of positive animals (%) |
|---|---|---|---|
| 2007.6–2008.3 | 21 | 0 | 0 |
| 2008.4–2009.3 | 68 | 0 | 0 |
| 2009.4–2010.3 | 136 | 2 | 1.5 |
| 2010.4–2011.3 | 123 | 5 | 4.1 |
| 2011.4–2012.3 | 71 | 2 | 2.8 |
| 2012.4–2013.3 | 101 | 7 | 6.9 |
| 2013.4–2014.3 | 134 | 32 | 23.9 |
| 2014.4–2015.3 | 184 | 69 | 37.5 |
| 2015.4–2016.3 | 276 | 138 | 50.0 |
| 2016.4–2017.3 | 368 | 215 | 58.4 |
| 2017.4–2018.3 | 437 | 234 | 53.5 |
| 2018.4–2019.3 | 380 | 212 | 55.8 |
| Total | 2,299 | 916 | 39.8 |

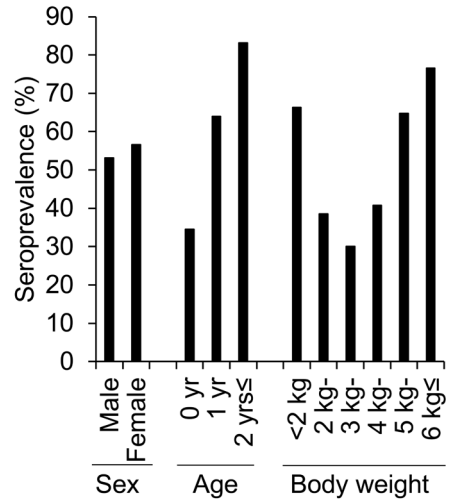


**Fig. 3.** Comparison of the seroprevalence of severe fever with thrombocytopenia syndrome virus (SFTSV) infection among raccoons captured in Tanabe region after April 2015 according to sex, age and body weight.

**Table 3.** Comparison of anti-severe fever with thrombocytopenia syndrome virus (SFTSV) antibody seroprevalence among raccoons in Tanabe region from April 2015 to March 2019

| | | Number of examined animals | Number of positive animals | Percentage of positive animals (%) |
|---|---|---|---|---|
| Sex | Male | 813 | 432 | 53.1 |
| | Female | 648 | 367 | 56.6 |
| Age[a,*] | 0 years | 655 | 226 | 34.5 |
| | 1 year | 508 | 325 | 64.0 |
| | 2 years≤ | 298 | 248 | 83.2 |
| Body weight* | <2 kg | 80 | 53 | 66.3 |
| | 2 kg- | 109 | 42 | 38.5 |
| | 3 kg- | 230 | 69 | 30.0 |
| | 4 kg- | 337 | 137 | 40.7 |
| | 5 kg- | 351 | 227 | 64.7 |
| | 6 kg- | 354 | 271 | 76.6 |
| Total | | 1,461 | 799 | 54.7 |

a: Age of each raccoon was classified into 3 age classes, 0, 1, and 2+, based on observation on canine pulp cavity, cranial suture and femur epiphysis. *Significant difference in the categories ($P<0.05$).

### *Virus isolation from raccoons*

To examine whether SFTSV was active in each sample, SFTSV was isolated from serum samples from two raccoons (TNB1580 and 1590), spleen, lymph node, and bladder in SRH186, and lung, small intestine, colon, spleen, lymph node, and bladder in SRH187 (Table 6).

### *Phylogenetic analysis*

A phylogenetic analysis with four isolates showed that TNB1580 and TNB1590 were clustered in the Chinese genotype C5, while SRH186 and SRH 187 were classified in Japanese genotype J1 (Fig. 4) [29]. In addition, short fragments amplified from raccoon sera were analyzed. As a result, 13 and 20 in Wakayama Prefecture were classified into two genotypes, J1 and C5, respectively (Table 7).

### *Histopathology and immunohistopathology*

To detect lesions and viral antigens by SFTSV infection, a histopathological examination was performed by H&E staining and

**Table 4.** Detection of severe fever with thrombocytopenia syndrome virus (SFTSV) gene in raccoon sera by RT-PCR from April 2013 to March 2019

| Month | Jan | Feb | Mar | Apr | May | Jun | Jul | Aug | Sep | Oct | Nov | Dec |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. of examined raccoons | 93 | 86 | 124 | 117 | 98 | 83 | 116 | 108 | 107 | 150 | 145 | 147 |
| No. of positive raccoons | 1 | 1 | 0 | 2 | 3 | 5 | 3 | 5 | 1 | 4 | 3 | 5 |
| Percentage of positive raccoons (one month) (%) | 1.1 | 1.2 | 0.0 | 1.7 | 3.1 | 6.0 | 2.6 | 4.6 | 0.9 | 2.7 | 2.1 | 3.4 |
| Percentage of positive raccoons (three months) (%) | | 0.7* | | | 3.4 | | | 2.7 | | | 2.7 | |

*Significant difference in comparison to Apr–Jun, Jul–Sep, and Oct–Dec.

**Table 5.** Comparison of prevalence of severe fever with thrombocytopenia syndrome virus (SFTSV) according to sex, age, and body weight

| | | Number of examined animals | Number of positive animals | Percentage of positive animals (%) |
|---|---|---|---|---|
| Sex | Male | 764 | 14 | 1.8 |
| | Female | 610 | 19 | 3.1 |
| Age | 0 years | 578 | 16 | 2.8 |
| | 1 year | 515 | 11 | 2.1 |
| | 2 years- | 281 | 6 | 2.1 |
| Body weight | <2 kg | 111 | 5 | 4.5 |
| | 2 kg- | 111 | 2 | 1.8 |
| | 3 kg- | 187 | 5 | 2.7 |
| | 4 kg- | 344 | 7 | 2.0 |
| | 5 kg- | 327 | 11 | 3.4 |
| | 6 kg- | 293 | 3 | 1.0 |
| | ND | 1 | 0 | 0 |
| Total | | 1,374 | 33 | 2.4 |

ND: no data.

immunostaining to detect SFTSV antigen in two raccoons (SRH186 and 187). There were no gross lesions and no clear histological changes in various organs from both raccoons. Only in SRH187, viral antigens were detected in the large mononuclear leukocytes in the submucosal lymphoid follicle of the colon, without any significant histological lesions (Fig. 5).

### *Detection of SFTSV genes in tissues of raccoons*

To compare the amount of SFTSV in each tissue, real-time RT-PCR using tissues of SFTSV-positive raccoons SRH186 and SRH187 showed that many specimens, including the brain, liver, kidney, lung, small intestine, colon, spleen, lymph node, bladder, trachea, feces and serum in SRH186 and brain, liver, kidney, lung, small intestine, colon, spleen, lymph node, trachea, and serum in SRH187 were positive (Table 7). Especially, the colon in SRH186 showed the highest number of genome copies.

## DISCUSSION

Wild animals must play important roles in the SFTSV transmission cycle. Some animals develop viremia due to SFTSV infection and SFTSV was transmitted to ticks by blood sucking. Some animals carry SFTSV-infecting ticks from endemic regions to non-endemic regions. In addition, since wild animals are more frequently infested by ticks than domestic animals and humans, the seroprevalence of SFTSV infection in wild animals can be expected to be higher than that in domestic animals and humans. Therefore, wild animals must be superior sentinels for risk assessment of SFTSV infection in humans. However, the prevalence of SFTSV infection in wild animals has been unclear. In this study, we examined SFTSV infection in feral raccoons.

We have many serum samples from raccoons captured in Tanabe region, Wakayama Prefecture, because an epidemic of canine distemper occurred in this region between 2007 and 2008 (Fig. 1) [23]. Since our first isolation of SFTSV in Japan in 2012 [24], we have examined seroprevalence of SFTSV infections by ELISA using these stored sera.

At first, we established an ELISA and compared with the results by the $FRNT_{50}$. Surprisingly, the specificity and sensitivity of the ELISA were both 100% when a cut-off value of 0.564 was applied (Table 1). This ELISA system was good for the detection of antibodies against SFTSV in raccoon sera. However, our preliminary data indicated that this ELISA system was not good for the surveillance of SFTSV infection in some other animals, because the specificity and sensitivity were low.

Next, mass surveillance of SFTSV infection in raccoons in Tanabe region was performed. The first anti-SFTSV antibody-

**Table 6.** Information on raccoons that were found to be positive for severe fever with thrombocytopenia syndrome virus (SFTSV) by RT-PCR or virus isolation in Wakayama Prefecture from April 2013 to March 2019

| Virus name | Date of sampling (d/m/y) | Age (years) | Body weight (kg) | Sex | Detection by RT-PCR or isolation | Genotype | DNA data bank of Japan (DDBJ) accession number |
|---|---|---|---|---|---|---|---|
| TNB1580 | 19/11/2013 | 0 | 5.5 | F | Isolation | C5 | LC579715-17 |
| TNB1586 | 4/12/2013 | 1 | 6.6 | M | RT-PCR | C5 | LC579728 |
| TNB1588 | 5/12/2013 | 0 | 5.3 | F | RT-PCR | C5 | LC579729 |
| TNB1590 | 9/12/2013 | 0 | 2.3 | F | Isolation | C5 | LC579718-20 |
| TNB1656 | 7/4/2014 | 1 | 4.5 | F | RT-PCR | J1 | LC579730 |
| TNB1668 | 25/4/2014 | 1 | 5.0 | M | RT-PCR | J1 | LC579731 |
| TNB1672 | 11/5/2014 | 2+ | 6.1 | M | RT-PCR | J1 | LC579732 |
| SRH186 | 13/6/2014 | 0 | 1.1 | F | Isolation | J1 | LC579712-14 |
| SRH187 | 13/6/2014 | 0 | 1.1 | F | Isolation | J1 | LC579709-11 |
| MNB463 | 28/6/2014 | 2+ | 5.4 | M | RT-PCR | J1 | LC579721 |
| TNB1684 | 7/7/2014 | 0 | 0.9 | F | RT-PCR | J1 | LC579733 |
| MNB465 | 22/7/2014 | 0 | 0.7 | F | RT-PCR | C5 | LC579722 |
| MNB466 | 4/8/2014 | 0 | 2.3 | F | RT-PCR | C5 | LC579723 |
| MNB467 | 4/8/2014 | 2+ | 5.9 | M | RT-PCR | C5 | LC579724 |
| SRH239 | 20/5/2015 | 2+ | 6.5 | M | RT-PCR | J1 | LC579725 |
| TNB1943 | 13/10/2015 | 2+ | 5.0 | F | RT-PCR | C5 | LC579734 |
| SRH283 | 27/10/2015 | 0 | 3.9 | F | RT-PCR | J1 | LC579726 |
| TNB1988 | 20/11/2015 | 0 | 4.2 | M | RT-PCR | J1 | LC579735 |
| TNB2016 | 11/12/2015 | 0 | 3.8 | M | RT-PCR | C5 | LC579736 |
| TNB2040 | 8/1/2016 | 0 | 5.1 | F | RT-PCR | J1 | LC579737 |
| SRH353 | 9/8/2016 | 1 | 5.9 | F | RT-PCR | J1 | LC579727 |
| TNB2256 | 1/9/2016 | 0 | 1.7 | F | RT-PCR | C5 | LC579738 |
| TNB2316 | 2/11/2016 | 1 | 5.9 | F | RT-PCR | C5 | LC579739 |
| TNB2405 | 2/12/2016 | 1 | 4.8 | M | RT-PCR | C5 | LC579740 |
| TNB2593 | 3/6/2017 | 1 | 3.5 | F | RT-PCR | C5 | LC579741 |
| TNB2612 | 18/7/2017 | 1 | 4.5 | M | RT-PCR | J1 | LC579742 |
| TNB2666 | 16/10/2017 | 1 | 5.2 | M | RT-PCR | C5 | LC579743 |
| TNB2923 | 10/2/2018 | 0 | 3.3 | F | RT-PCR | C5 | LC579744 |
| TNB3068 | 10/5/2018 | 1 | 4.2 | F | RT-PCR | C5 | LC579745 |
| TNB3079 | 4/6/2018 | 2+ | 5.4 | M | RT-PCR | C5 | LC579746 |
| TNB3132 | 2/8/2018 | 1 | 5.3 | F | RT-PCR | C5 | LC579747 |
| TNB3138 | 10/8/2018 | 0 | 4.8 | M | RT-PCR | C5 | LC579748 |
| TNB3161 | 1/10/2019 | 0 | 3.1 | M | RT-PCR | C5 | LC579749 |

positive raccoons were captured in October 2009. From April 2009 to March 2013, the seroprevalence was <10%, but rapidly increased from April 2013. After April 2015, the seroprevalence reached >50%. Importantly, the first patient in this region was reported in June 2014, with SFTS patients reported each year since that time [19]. These results suggested that the expansion of SFTSV among wild animals is a risk factor for SFTSV infection in humans. In other words, the surveillance of SFTSV infection among wild animals can be used to assess the risk in humans.

Since April 2015, 54.7% (799/1,461) of raccoons in Tanabe region had a history of SFTSV infection (Table 2). Interestingly, many raccoons with a body weight of less than 2 kg (66.3%) possessed anti-SFTSV antibodies, while only 38.5% of raccoons with a body weight of 2–3 kg possessed anti-SFTSV antibodies. This result in pups was similar to the results (71.1%) observed in raccoons of ≥1 year of age, indicating that many pups acquired maternal antibodies from their mothers and that these pups would be protected from SFTSV infection. In raccoons with a body weight of ≥3 kg, the seroprevalence of SFTSV infection rapidly increased in a body weight-dependent manner (Fig. 3, Table 3). Similarly, the seroprevalence increased in an age-dependent manner. These results indicated that many raccoons were infected with SFTSV after the loss of maternal antibodies and most adult raccoons (≥1 year of age) had anti-SFTSV antibodies. Similarly, the SFTSV genome was detected in many raccoons of <1 year of age (Table 6).

RT-PCR revealed that 2.4% of raccoons were infected with SFTSV at their capture and that these SFTSV-positive raccoons seemed to be healthy. In addition, many raccoons possessed antibodies against SFTSV, indicating that they had recovered from SFTSV infection. These results indicated that raccoons are infected with SFTSV, but that most do not show clinical signs. We succeeded in collecting tissue samples from two SFTSV-positive raccoons and SFTSV genes were detected in many samples, including feces and serum (Table 7), suggesting that SFTSV was secreted from healthy raccoons. In addition, the lack of clear pathological change in any tissue specimens from two infected raccoons (Fig. 5) also supported that raccoons with SFTSV infection may not show severe clinical signs. SFTSV was transmitted from the body waste of some human SFTS patients [14].

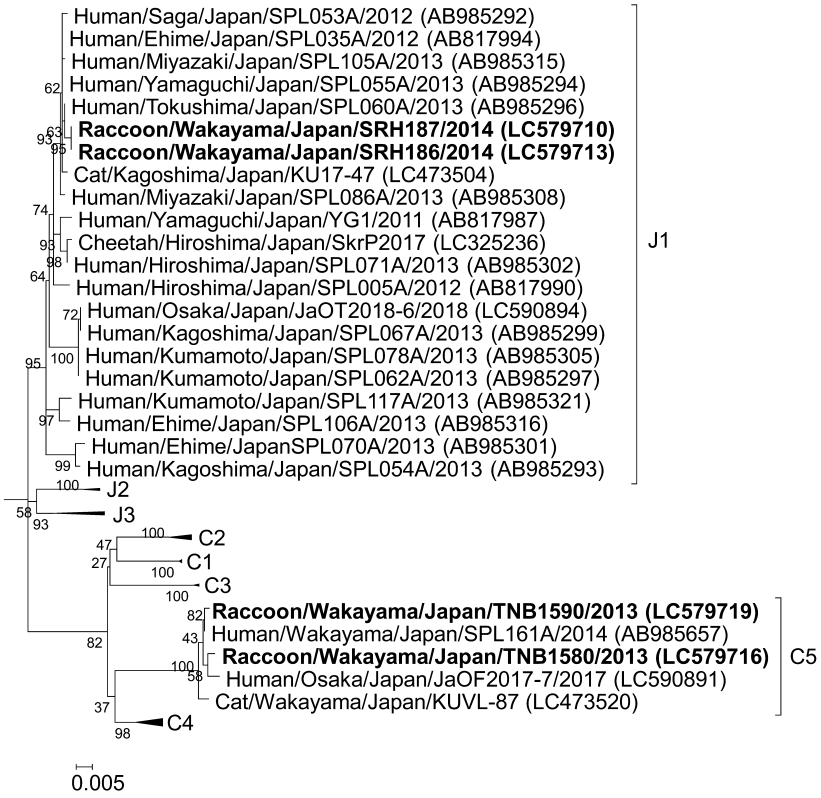


**Fig. 4.** Phylogenetic analysis of severe fever with thrombocytopenia syndrome virus (SFTSV) strains isolated from raccoons. The nucleotide sequences obtained from four raccoons were aligned with 45 other sequences deposited in the DNA data bank of Japan (DDBJ) using Clustal W and a phylogenetic tree was constructed by the Maximum likelihood method based on the General Time Reversible model using the MEGA 7.0.26 software program. The confidence of the tree was evaluated by 1,000 bootstrap replications. Genotypes J1–J3 and C1–C5 were reported by Yoshikawa *et al.* [29]. Our isolates are indicated by bold typeface. The DDBJ accession numbers are shown in parentheses. Guertu virus (NCBI accession number: NC_043609) was used as an outgroup to construct phylogenetic tree [1].

**Table 7.** Detection of severe fever with thrombocytopenia syndrome virus (SFTSV) gene and virus isolation in raccoon tissue specimens

| Tissues | RT-PCR | | Virus isolation | |
|---|---|---|---|---|
| | SRH186 | SRH187 | SRH186 | SRH187 |
| Brain | + | + | - | + |
| Liver | ++ | ++ | - | - |
| Kidney | + | + | - | - |
| Lung | ++ | ++ | - | + |
| Small intestine | ++ | ++ | - | + |
| Colon | +++ | ++ | - | + |
| Spleen | ++ | ++ | + | + |
| Lymph node | ++ | ++ | + | + |
| Bladder | + | - | + | + |
| Trachea | ++ | + | - | - |
| Feces | + | - | ND | ND |
| Serum | + | + | - | - |

-: Cp value in real-time RT-PCR is >40, +: Cp value is 35–39, ++: Cp value is 30–34, +++: Cp value is 25–29, ND: not done.

Thus, care may be required in handling feces of SFTSV-infected raccoons due to the possibility of SFTSV infection; however, there are no reports on SFTSV infection from animal feces. Since many raccoons in Japan are captured as an invasive species by local governments, individuals who trap raccoons should also take measures to protect themselves from SFTSV infection due to contact with body fluids.

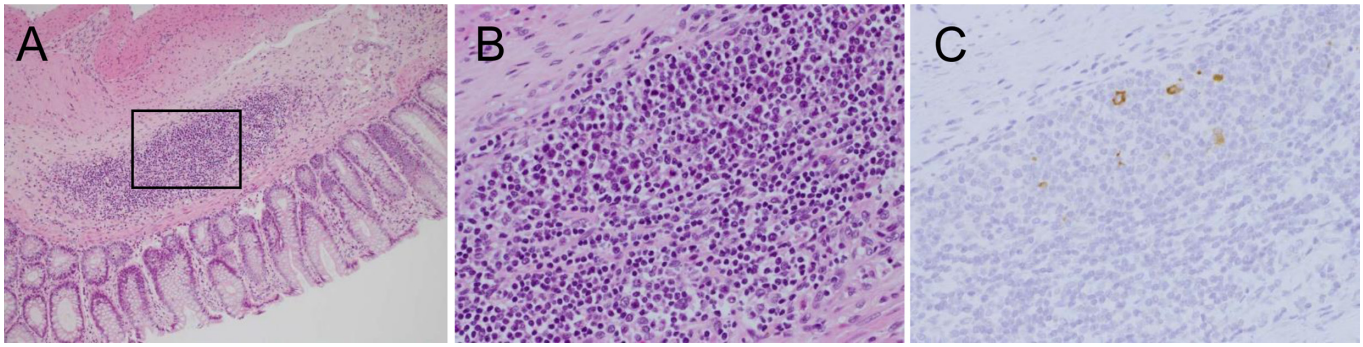


**Fig. 5.** Histopathological and immunohistochemical examinations of the colon of a raccoon (SRH187) infected with severe fever with thrombocytopenia syndrome virus (SFTSV). Lymphoid cells were positive for SFTSV-NP antigen. (**A**) Hematoxylin and eosin (H&E) staining of the colon showed no pathological changes (original magnification ×100) (**B**) H&E staining of submucosal lymphoid follicle in the colon (original magnification ×400) (**C**) Immunohistochemical (IHC) staining of SFTSV-NP antigen in lymphoid cells in lymphoid follicle (original magnification ×400).

SFTSV genes were detected from many raccoons and the ratio of detection was significantly different among the seasons. The ratio was lowest (0.7%) in winter and highest (3.4%) in spring (Table 4). This may be due to the fact that many tick species in Japan are less active in winter than in spring. Similarly, the number of human SFTS cases in Japan was highest in May. The situation of SFTSV infection among raccoons seems to influence the risk of SFTSV infection in humans.

In this area, two genotypes, J1 and C5, are spreading among raccoons (Table 6, Fig. 4). Raccoons with genotype C5 infection show very similar manifestations to human SFTS patients in Wakayama Prefecture, suggesting that humans and raccoons are infected with the same viruses. Thus, SFTSV must spill-over to humans from circulation among wild animals and ticks.

In conclusion, many raccoons in the field were infected with SFTSV and the raccoon is a useful sentinel for assessing the risk of SFTSV infection in humans. In addition, in many raccoons in endemic areas, SFTSV infection is not apparent, and these animals move around human spaces, suggesting that raccoons with viremia may be an important reservoir for SFTSV in humans. The further analysis of SFTSV infection in other wild animals will be required to determine the most important reservoirs for SFTSV.

CONFLICTS OF INTEREST. The authors declare no conflicts of interest in association with the present study.

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