Epidemiological studies on severe fever with thrombocytopenia syndrome virus among wild animals

重症熱性血小板減少症候群ウイルスの野生動物における疫学調査

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1. General Introduction

1.1. History

Severe fever with thrombocytopenia syndrome virus (SFTSV) causes hemorrhagic fever and gastrointestinal symptom in human as new emerging infectious disease (Takahashi et al., 2013). In 2009, unknown disease emerged in rural area in Hubei province, China, following patients suspected to be anaplasmosis. In 2011, by analysis of isolate derived from the patient's blood, the agent was identified as a novel bunyavirus, SFTSV (Yu et al., 2011). At the same time, the report indicated possibility as tick-borne pathogen, because viral genes were detected from *Heamophysalis longicornis* and phylogenetically closed to tick-borne viruses. However, the retrospective study in 2012 in China reported the case of patients were infected by the contact with discharge from SFTS patients without history of tick-bite (Yu et al., 2012).

In Japan, first case was reported in 2012 in Yamaguchi (Takahashi et al., 2013). The dead patient was suspected to viral infection with hemophagocytic syndrome and disseminated intravascular coagulation. The causative agent in the patient was identified as SFTSV by virus isolation in Yamaguchi university. Since the first SFTS patient was reported, several patients were diagnosed as SFTS retrospectively. Subsequently, the distribution of SFTS patients has been found in Korea, Vietnam and Taiwan in 2013, 2018 and 2019 (Kim et al., 2013; Tran et al., 2019; Peng et al., 2020).

In 2012, antibodies against SFTSV were detected from several live stocks and wild animals in China, indicating that SFTSV has wide host range among animals (Jiao et al., 2012). In 2013, further serosurvey showed high seropositivities of antibody in livestocks in endemic areas (Niu et al., 2013). The SFTSV from animals were phylogenetically close to SFTSV derived from SFTS patients and ticks (Niu et al., 2013).

In 2015, it was noted ticks could be vector and reservoir of SFTSV, since tick

received virus from infected animals and passed it transstadially and transovarially (Luo et al., 2015). The infected ticks also transmitted the virus to animal (Luo et al., 2015).

In 2017, it was reported that domestic cats infected with SFTSV showed severe clinical signs with high mortality rate similar to SFTS patients (Matusu et al., 2019). Notably, since 2017, direct transmission cases of human by diseased cats and dogs have been reported in Japan and SFTSV infection from pet animals has been important public health issue (Kida et al., 2019; Yamanaka,.2020; Ando et al.,2021; Miyauchi et al.,2022; Oshima et al.,2022; Kirino et al., 2022; Kobayashi et al., 2020).

1.2. Virus properties

SFTSV is negative-stranded and triple-segmented RNA virus. SFTSV belongs to order *Bunyavirinae* family *Phenuiviridae* genus *Bandavirus*. SFTSV was classified as Dabie bandavirus by international committee on taxonomy of viruses (ICTV) in 2020. However, the virus is formally called as SFTSV.

SFTSV genome is consisted of three segments, small (S) segment, medium (M) segment and large (L) segment, containing approximately 6.4k, 3.4k and 1.7kb, respectively. The sequences of 3' and 5' ends of segments are complementary form pan-handle structure (Yu et al., 2011).

L segment encodes RNA-dependent RNA polymerase (RdRp), M segment encodes glycoprotein precursor (GPC) and S segment is an ambisense gene encoding nucleocapsid (N) protein in negative sense and nonstructural (NSs) protein in positive sense (Yu et al., 2011).

GPC is cleaved into two viral envelope glycoproteins (Plegge et al., 2016), Gn and Gc which are formed spike complex to be Glycoprotein (GP) localized in the ER or

Golgi apparatus (Tani et al,. 2014; Plegge et al., 2016). The GP associates to host cell entry (Tani et al., 2014). The GP was targeted by virus-neutralization antibody and inhibited to cell entry (Hofmann et al., 2013; Tani et al., 2014). C-type lectin and dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) are recognized as an initial binding receptor for SFTSV (Hofmann et al., 2013)

NSs protein is associated with the suppression of host innate immunity as antagonist targeting the IFN response pathway (Chaudhary et al.,2015; Yoshikawa et al.,2019; Moriyama et al., 2018). In more detail, NSs could bind to human STAT-1 and STAT-2 and avoids JAK-STAT passway which activates IFN signaling pathway by forming viral inclusion bodies in cytoplasmic region with STAT-1 and STAT-2 (Ning et al., 2015; Chaudhary et al.,2015). On the other hand, NSs didn't bind to murine STAT-2, resulting in the loss pathogenesis of SFTSV in immunocompetent mouse model. (Yoshikawa et al.,2019).

N protein interacts with viral RNA together with RdRp and forms the ribonucleoprotein (RNP) complex (Lokupathirage et al.,2021). N protein contributes to localization of RNP in the Golgi apparatus and endoplasmic reticulum-Golgi intermediate compartment (ERGIC) and viral assembly. (Lokupathirage et al., 2021). Since N protein is highly conserved genetically, N protein is often targeted for developing immunological diagnosis method (Jiao et al., 2012; Yu et al., 2015).

RdRp associates to replication of viral genomic RNA. As described above, RdRp forms RNP complex with N protein and viral genome (Lokupathirage et al., 2021). RdRp is the antiviral target by favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) which is a candidate of antiviral drug with inhibition of replication of viral genome (Furuta et al., 2017; Tani et al., 2016; Saijo et al., 2018).

1.3. Epidemiology and transmission cycle

SFTSV distributes in East Asia and most patients with SFTS have been reported in China, Korea and Japan, while only 2 and 1 patients were reported in Vietnam and Taiwan, respectively (Miao et al., 2021; Kobayashi et al., 2020; Yun et al., 2020; Tran et al., 2019; Peng et al., 2020).

In Japan, the number of SFTS patient reported by the years during 2013-2021 were 48, 61, 60, 60, 90, 77, 101, and 78, respectively (Infectious diseases weekly report (IDWR) by national institute of infectious diseases (NIID)). The case fatality rates (CFR) (27%) in Japan is higher than 10% in China and 22% Korea (Miao et al., 2021; Kobayashi et al., 2020; Yun et al., 2020). The oldest SFTS patient in Japan was discovered in 2005 in Nagasaki (Kurihara et al., 2016). The SFTS cases mainly distribute in western part of Japan (Yoshikawa et al., 2015; Kobayashi et al., 2020). On the other hand, the endemic region has been expanded gradually to the center of Japan (Kobayashi et al., 2020). Most patients were reported during April to October. The seasonality of SFTS patients is consisted with the seasonal activity of ticks from spring to autumn (Kobayashi et al., 2020; Kato et al., 2016; Lee et al., 2021). In serosurveillance, 8.4 to 0.4% of healthy people in China and 0.14% in Japan possessed anti-SFTSV antibody in endemic area (Cui et al., 2013; Liang et al., 2013; Li et al., 2014; Kimura et al., 2018).

It was indicated that SFTSV are phylogenetically classified into two large groups which is Japanese clade and Chinese clade (Yoshikawa et al., 2015). The two group are additionally grouped into Japanese clade 1, 2 and 3, or Chinese clade 1,2,3,4 and 5 and the groups are consistent with geographical distribution (Yoshikawa et al., 2015). However, several SFTSV in Chinese clade were reported in Japan (Yoshikawa et

al., 2015; Ikemori et al., 2021). Both of Japanese and Chinese clades of SFTSV distribute in Republic Korea, indicating possibilities of transmission over countries (Yun et al., 2015; Yun et al., 2020a; Yun et al., 2020b). And migratory birds might contribute to transboundary movement of ticks with SFTSV (Yun et al., 2015).

SFTSV has been detected in various tick species (Wang et al., 2015; Yun et al., 2016; Lin et al., 2020; Sato et al., 2021). Especially, *Haemophysalis longicornis* is considered to be a major vector and reservoir of SFTSV (Yu et al., 2015; Wang et al., 2015). The viral sequences derived from ticks showed high homology with isolates from human and animals (Wang et al., 2015). *H. longicornis* transmitted the virus transstadionaly and transovarialy to next stages of ticks (Luo et al., 2015; Zhuang et al., 2018). Besides, ticks are received virus from infected animals by blood-sucking and SFTSV-infected ticks transmit virus to animal. (Luo et al., 2015; Zhuang et al., 2018).

SFTSV RNA has been detected in various animals, including cattle (Niu et al., 2013; Xing et al., 2016), goats (Niu et al., 2013; Xing et al., 2016), sheep (Niu et al., 2013; Lin et al., 2020), pigs (Niu et al., 2013), chicken (Niu et al., 2013), dogs (Niu et al., 2013; Matsuu et al., 2021), cats (Matsuu et al., 2019), cheetahs (Matsuno et al., 2018), water deer (Oh et al., 2016; Lee et al., 2021), wild boar (Oh et al., 2016; Rim et al., 2021; Matsuu et al., 2021; Kirino., 2021), mongooses (Kuba et al ., 2021), shrews (Liu., 2014), and rodents (Liu., 2014). SFTSV detected gene from animals always shows highly homology with isolates from human patients in same area (Li et al., 2016; Niu et al., 2013).

Seroprevalence of anti-SFTSV antibody has been reported in domestic animals, including cattle (Niu et al., 2013; Xing et al., 2016; Ding., 2014; Li et al., 2014; Tabara et al., 2016), goats (Cui et al., 2013; Xing et al., 2016; Ding., 2014; Li et al., 2014;Kang et

al., 2018), sheep (Niu et al., 2013; Lin et al., 2020), dogs (Niu et al., 2013; Cui et al., 2013; Ding., 2014; Li et al., 2014 ; Lee et al., 2017; Kimura et al., 2018; Matsuu et al., 2021; Hashimoto et al., 2022), cats (Lee et al., 2017;Matsuu et al., 2021; Hashimoto et al., 2022), pigs (Niu et al., 2013; Li et al., 2014), chickens (Niu et al., 2013; Ding., 2014; Li et al., 2014), geeses (Li et al., 2014) and minks (Wang., 2017), and in wild animals including wild boars (Hayasaka et al., 2016; Rim et al., 2021; Oh et al., 2016; Matsuu et al., 2021; Hashimoto et al., 2022; Kimura et al., 2018), deer (Matsuu et al., 2021; Hashimoto et al., 2022; Kimura et al., 2018), water deer (Oh et al., 2016), mongooses (Kuba et al ., 2021), hedgehogs (; Li et al., 2014; Sun., 2017), shrews (Liu., 2014) and rodents (Li et al., 2014; Liu., 2014).

In China, domesticated animals such as cattle, sheep and goat were suspected to be amplifiers of SFTSV in endemic area (Niu et al., 2013). On the other hands, lower seroprevalence was observed in pigs than other domesticated animals (Niu et al., 2013; Li et al., 2014).

The epidemiological study in Japan indicated very low seroprevalence (2.2%) in cattle in Shimane (Tabara et al., 2016). On the other hand, serological surveillance among wild animals, Sika deer and wild boars in Japan indicates high seroprevalence in endemic area (Matsuu et al., 2021; Hayasaka et al., 2016; Hashimoto et al., 2022; Kimura et al., 2018). Wild animals must play important role in transmission cycles of SFTSV in nature.

As describe above, infection of SFTSV is lethal for most cats (Matsuu et al., 2019; Park et al., 2021). And less seroprevalence were observed than the other domesticated and wild animals (Matsuu et al., 2021). Therefore, cats must be highly sensitive for SFTSV and many cats should be dead by SFTSV infection.

1.4. Diseases and pathogenesis

SFTSV infection to human causes severe symptom with high mortality (Kobayashi et al., 2020). Patients mostly shows gastrointestinal symptom, leukopenia and thrombocytopenia such as hemorrhagic symptom, because of disseminated intravascular coagulation and hemophagocytosis (Takahashi et al., 2013; Kobayashi et al., 2020). The mortality in SFTS patients showed 27% in Japan (Kobayashi et al., 2020).

Infected cats with SFTSV shows acute and severe clinical feature including anorexia, fever and vomiting with 62.5% of the case fatality rate (Matsuu et al., 2019; Sakai et al., 2021). Animal experiment revealed that challenge with SFTSV could be lethal infection in cats with severe hemorrhaging on their gastrointestinal tract following high viral shedding in body waste. (Park et al., 2019).

Cheetahs were also susceptible for SFTSV infection (Matsuno et al.,2018). Two captive cheetahs showed severe symptoms like SFTS in human and dead (Matsuno et al.,2018).

SFTSV infection in ferret also showed age-dependent pathogenicity (Park et al., 2019). Immunocompetent mouse (Liu et al., 2016; Chen et al., 2012; Jin et al., 2012), hamster (Gowen et al., 2016) and monkey (Matsuno et al., 2017; Jin et al., 2015) exhibited asymptomatic or moderate disease after SFTSV infection. Alpha/beta interferon receptor knockout mice were susceptible to SFTSV infection, which are employed as lethal animal models of SFTSV infection (Liu et al., 2014; Tani et al., 2016; Tani et al., 2018; Matsuno et al., 2017).

1.5. Diagnosis

SFTS in human was included in Japan’s Infectious Diseases Control Law as a

category IV notifiable disease. The diagnosed SFTS patients must be reported to the National Epidemiological Surveillance of Infectious Diseases (NESID) operated by the National Institute of Infectious Diseases (NIID). For diagnosis in human, gene detection or isolation of SFTSV are performed in acute phase. Furthermore, antibody tests by ELISA, IFA and VN test are also performed using pre and post serum of patient.

To isolate SFTSV, DH82 cells were used in first report in China (Yu., 2011). Additionally, Vero cells is also employed for isolation of infectious virus in Japan (Yoshikawa et al., 2015; Takahashi et al., 2013).

For seroserveillance in Japan, ELISA using lysate of infected cell were applied by using sera from human and animals (Kimura et al., 2018; Uchida et al., 2018; Okada et al., 2021). Furthermore, Gaussia luciferase immunoprecipitation system (GLIPS) using cells expressing a recombinant N protein and indirect IgG ELISA using recombinant N protein were also established in Japan (Hayasaka et al.,2016; Matsuu et al., 2021). Comparably, for epidemiology of SFTSV infection among wild animals in China, double antigens ELISA using expressed N protein were mainly performed to detect antibodies from various animals (Niu et al., 2013; Jiao et al., 2012). The double antigen ELISA system could detect antibody against an SFTSV-like novel pathogen in USA (Xing Z et al., 2013).

For diagnosis in SFTS-suspected animals, RT-PCR system was developed and recommended using SFTSV specific primers (S2-200: 5'-GACACAAAGTTCATCATTGTCTTTGCCCT-3', S2-360: 5'-TGCTGCAGCACATGTCCAAGTGG-3') (Park et al., 2021). For rapid diagnosis of SFTS patients, the reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay to detect viral RNA was developed (Sano et al., 2021).

1.6. Treatment and Prevention

Currently, there are no available specific treatment and preventive measure against SFTSV infection in Japan. Favipiravir treatment showed effectiveness *in vitro* and *in vivo* (Furuta et al., 2017; Tani et al., 2016; Saijo et al., 2018). The favipiravir inhibited the replication of SFTSV in Vero cells and improved survival rates in IFNAR−/− mice infected with lethal challenge of SFTSV (Tani et al., 2016). At this stage, the efficacy of treatment of favipiravir to SFTS patient has been evaluated in clinical trials in Japan (Saijo et al., 2018). In China, clinical trial indicated that favipiravir treatment for the group of low-viral load patients significantly reduced CFR from 11.5 to 1.6%, but not for the group of high-viral load patients (Li et al., 2021).

Vaccine is considered to be one of the best measures for prevention of SFTS. Studies about vaccine were performed based on live-attenuated vaccine (Yu et al., 2019), viral vector vaccine (Yoshikawa et al., 2019; Dong et al., 2019; Tian et al., 2021), or DNA vaccine (Kwak et al., 2019; Kang et al., 2020). However, since nonhuman primate models of lethal SFTS have not been developed, the evaluation of candidates of vaccine has been difficult until now (Yoshikawa et al., 2021).

It is difficult to eliminate the risk of spill-over of tick-borne pathogens from transmission cycle in nature. However, treatment with insecticide and wearing the long sleeves and long pants to prevent the tick-bite are effective for reduction of the risk of SFTSV infection. The development of specific therapy and preventive measures against SFTSV infection is required strongly in the field of human and animal medicines.

2. Chapter 1

Roles of raccoons in the transmission cycle of SFTSV

2.1. 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.

2.2. Introduction

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 (Lin et al., 2020; Park et al. 2021; Takahashi et al., 2013; Tran et al., 2019; Yu et al., 2011). 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 (Kato et al., 2016; Kobayashi et al., 2020)

SFTS is known as a tick-borne zoonosis and to circulate between ticks and mammals, including domestic and wild animals (Li et al., 2016; Niu et al., 2013; Oh et al., 2016; Wang et al., 2015). 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 (Chen et al., 2019; Cui et al., 2013; Niu et al., 2013). On the other hand, clinical signs due to SFTSV infection have also been reported in zoo and domestic animals in Japan (Matsuno et al., 2019; Matsuu et al., 2019; Park et al., 2019). Surveillance showed that both antibodies and viral RNA could be detected from several wild animals, including wild boar, deer, hedgehogs, and rodents (Casel et al., 2021; Chen et al., 2019; Hayasaka et al., 2016; Li et al., 2016). In addition, it is suggested that migratory birds might carry SFTSV-infected ticks and contribute to the transboundary spreading of SFTSV (Yoshikawa et al., 2015; Yun et al., 2015). 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.

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 (Hayasaka et al., 2016; Kimura et al., 2018; Oh et al., 2016). 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.

2.3. Material and methods

2.3.1. 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, U.S.A.) 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 (Yu et al., 2011). The virus was propagated in Vero cells and stored at –80°C until use.

2.3.2. 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 hunted or 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 hours after death and the latest sampling was within 5 hours. Body weight was measured at 0.1 kg using spring scale. Age was determined 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.

2.3.3. Enzyme-linked immunosorbent assay (ELISA)

To determine the specific antibody against SFTSV in animal sera, enzyme-linked immunosorbent assay (ELISA) was performed using extracts from SFTSV HB29- or mock-infected HuH-7 cells (Fukuma et al., 2016; Kimura et al., 2016). In brief, antigens were coated with coating buffer (0.05M 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 Corporation, 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, U.S.A.) and the optical density (OD) was measured by a microplate reader (Bio-Rad, Hercules, CA, U.S.A.) using a 405 nm filter. For raccoons, an ELISA cut-off value of 0.564 was applied after comparison with a virus-neutralizing test.

2.3.4. 50% 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, U.S.A.). Infected cells were stained by rabbit antibodies against SFTSV N protein (Taniguchi et al., 2016) and Protein A/G conjugated with HRP (Thermo Fisher Scientific) using 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB; FUJIFILM Wako Pure Chemical Corporation). Serum samples that reduced the number of focuses to ≤50% of the number in control wells were considered to be positive.

2.3.5. 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) (Kanda et al., 2013). The sensitivity and specificity were estimated by the $FRNT_{50}$ as the gold standard.

2.3.6. 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.

2.3.7. 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.

2.3.8. 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') (Park et al., 2021). 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, Kanagawa, Japan). The sequences were deposited into the DNA Data Bank of Japan (DDBJ; Accession numbers: LC579721-579749).

2.3.9. 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, Shiga, Japan) and StepOne Real-Time RT-PCR System (Thermo Fisher Scientific), under the following conditions: 1 cycle of 42ºC for 5 min and 95ºC for 10 sec and 50 cycles of 95ºC for 5 sec, and 64ºC for 60 sec. The copy numbers were calculated using StepOne Software V2.1 (Thermo Fisher Scientific).

2.3.10. 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, U.S.A.). Genomic DNA libraries for each of the strains were constructed using NEBNextUltra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, U.S.A.) 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).

2.3.11. 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 (Kumer et al., 2016). 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 (Ando et al., 2021).

2.3.12. 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 (Tani et al., 2016).

2.3.13. Statistical analysis

The χ^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.

2.4. Results

2.4.1. Establishment of the ELISA for the detection of anti-SFTSV antibodies in raccoons

We determined exact the 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.

2.4.2. 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.

2.4.3. 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).

2.4.4. Virus isolation from raccoons

To examine whether SFTSV was active virus 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).

2.4.5. 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) (Yoshikawa et al., 2015). In addition, short fragments amplified from raccoon sera were analyzed. As a result, 13 and 20 SFTSV in raccoons in Wakayama prefecture were classified into two genotypes, J1 and C5, respectively (Table 7).

2.4.6. Histopathology and immunohistopathology

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

2.4.7. 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.

2.5. 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) (Suzuki et al., 2015). Since our first isolation of SFTSV in Japan in 2012 (Takahashi et al., 2013), we have examined seroprevalence of SFTSV infections by ELISA using these stored sera.

At first, we established an ELISA and compared the results obtained that 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-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 (Ohagi et al., 2014). 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% (799/1461) 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 (Liu 2012). 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.

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.

2.6. Legends for figures

Fig.1. Map of the SFTSV-endemic areas in Japan. Graduation of white to black indicates the number of human cases in the prefecture until 2020 (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.

Fig.2. Change in the seroprevalence of SFTSV infection among raccoons in Tanabe region (n=2,299) by year.

Fig. 3. Comparison of the seroprevalence of SFTSV infection among raccoons captured in Tanabe region after April 2015 according to sex, age and body weight.

Fig. 4. Phylogenetic analysis of SFTSV strains isolated from raccoons. The nucleotide sequences obtained from 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. The confidence of the tree was evaluated by 1,000 bootstrap replications. Genotypes J1-J3 and C1-C5 were reported (Yoshikawa et al.,2015). 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 (Ando et al., 2021).

Fig. 5. Histopathological and immunohistochemical examinations of the colon of a raccoon (SRH187) infected with 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).

2.7. Figures and tables

Fig.1

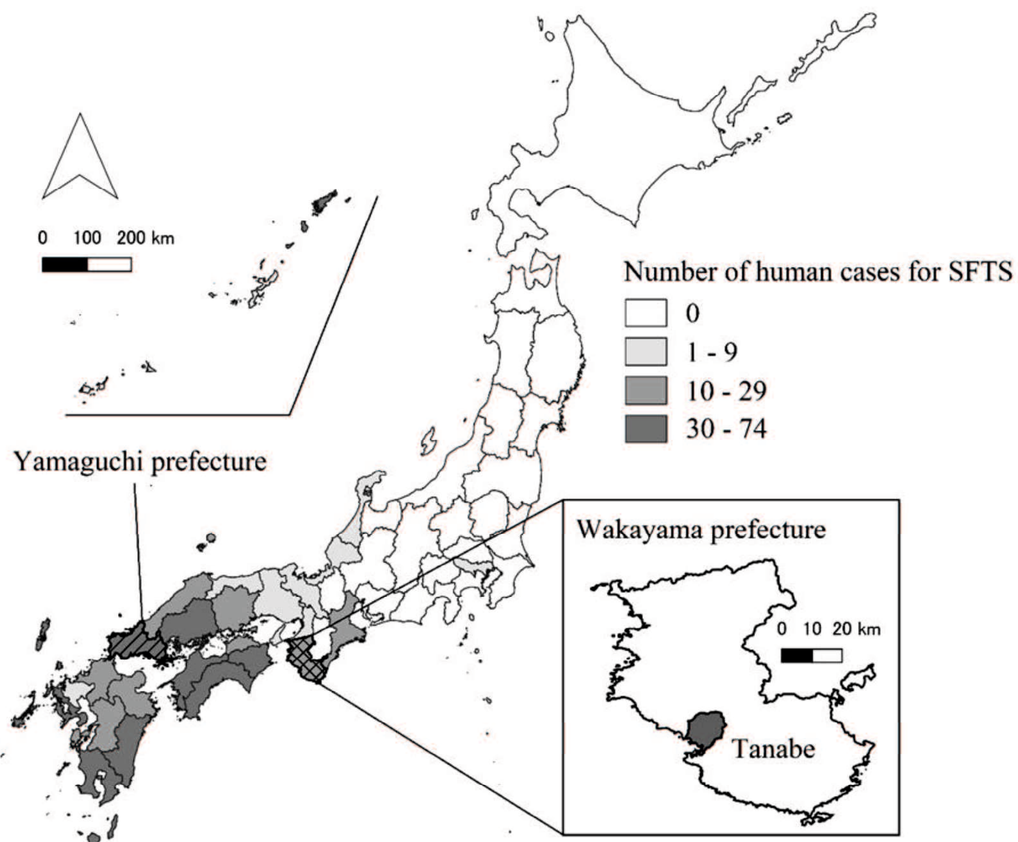

Fig.2

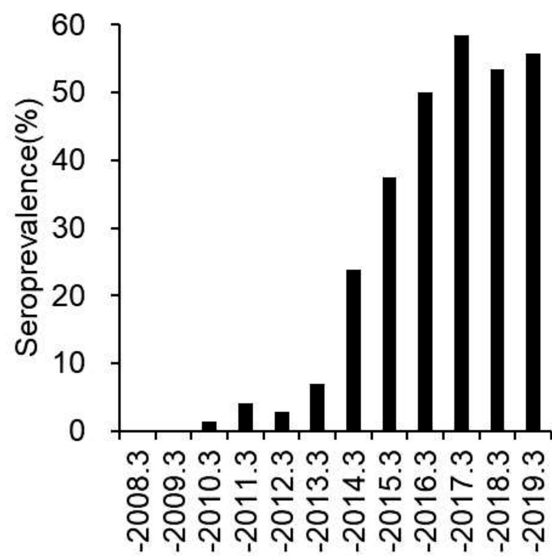

Fig.3

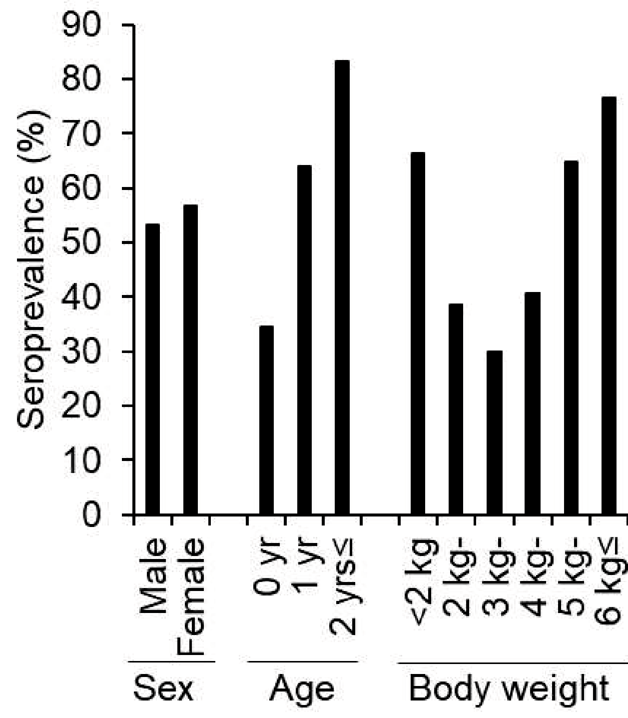

Fig.4

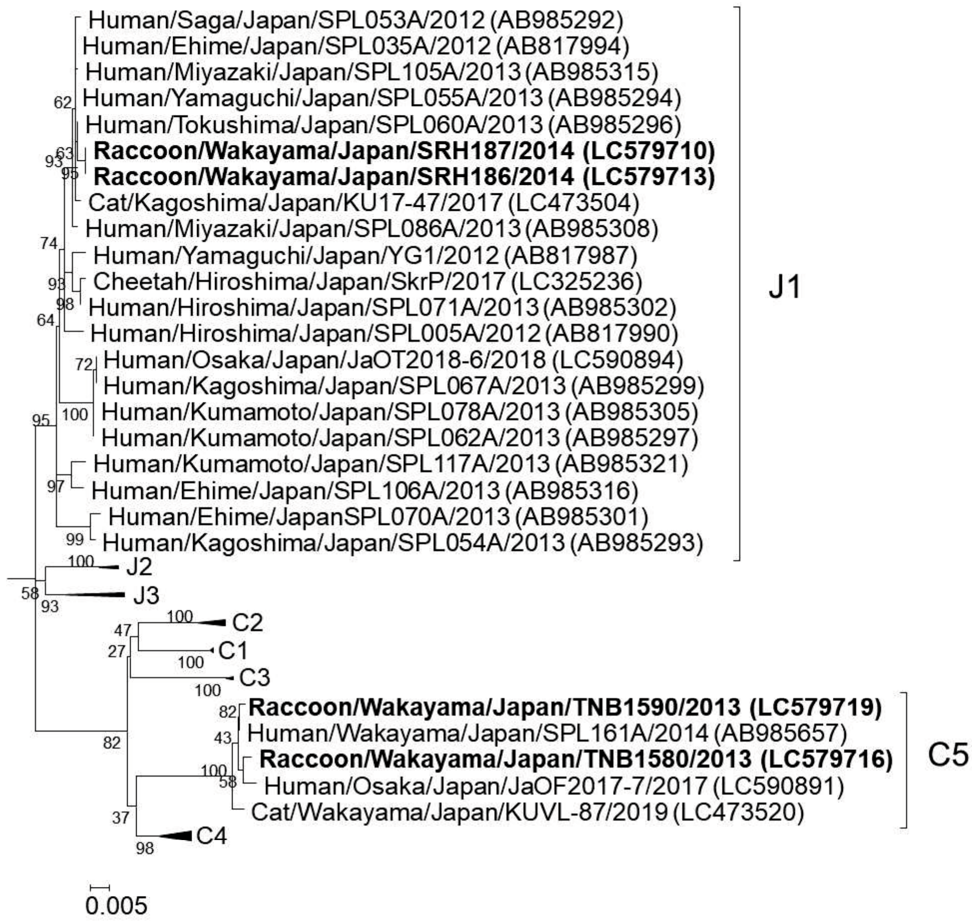

Fig.5

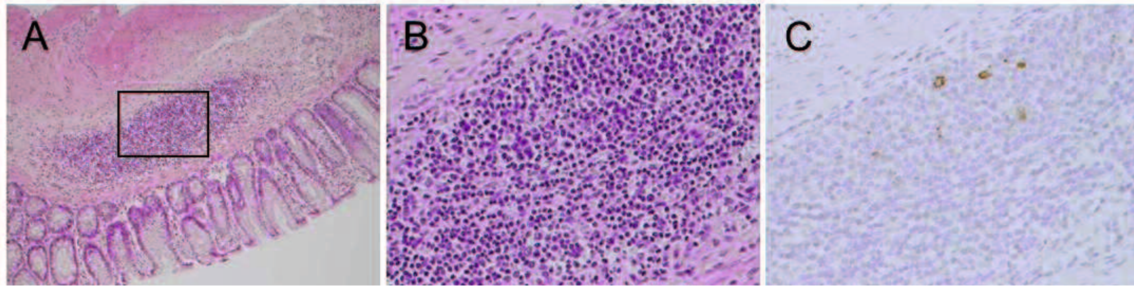

Table 1. Comparison of results between ELISA and $FRNT_{50}$

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

Table 2. Prevalence of anti-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	2299	916	39.8%

Table 3. Comparison of anti-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%
	2kg-	109	42	38.5%
	3kg-	230	69	30.0%
	4kg-	337	137	40.7%
	5kg-	351	227	64.7%
	6kg-	354	271	76.6%
Total		1461	799	54.7%

a: Age of each raccoon was determined 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 ($p<0.05$)

Table 4. Detection of SFTSV gene by RT-PCR in raccoon sera 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 with groups of Apr-Jun, Jul-Sep, and Oct-Dec.

Table 5. Comparison of prevalence of 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%
	2kg-	111	2	1.8%
	3kg-	187	5	2.7%
	4kg-	344	7	2.0%
	5kg-	327	11	3.4%
	6kg-	293	3	1.0%
	ND*	1	0	0%
Total		1374	33	2.4%

Table 6. Information on raccoons that were found to be positive for 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	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	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	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

Table 7. Detection of SFTSV gene and virus isolation from tissues of raccoons

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 cycles
ND: Not done

3. Chapter 2

Risk assessment of infection with SFTSV by 10-years serosurveillance of Sika deer and wild boar in Yamaguchi Prefecture

3.1. Abstract

In Japan, the first patient with severe fever with thrombocytopenia syndrome (SFTS) was reported from Yamaguchi Prefecture in 2012. In this study, retrospective surveillance of SFTSV infection in Sika deer and wild boar in this region over the 10-year period from 2010 to 2020 was conducted using a virus-neutralizing test. As a result, 510 of 789 Sika deer (64.6%) and 199 of 517 wild boars (38.5%) were positive for anti-SFTSV antibody. Interestingly, the seroprevalence in Sika deer significantly increased from 2010-2013 (42.4%) to 2015-2020 (81.0%). A similar tendency was observed in wild boar. The SFTSV gene was detected in 1 of 229 serum samples collected from Sika deer (0.4%), but not in 116 serum samples from wild boar. In conclusion, Sika deer may be more sensitive to SFTSV infection than wild boar, and surveillance of SFTSV infection in Sika deer could be used to assess the risk of SFTSV infection in humans.

3.2. Introduction

Severe fever with thrombocytopenia syndrome virus (SFTSV) is a triple-segmented and negative-stranded RNA virus that belongs to family *Phenuiviridae* genus *Bandavirus*. The virus is transmitted by tick bites and direct contact with discharges from SFTS patients and animals (Kato et al., 2016; Kobayashi et al., 2020; Liu et al., 2012; Miyauchi et al., 2022. SFTSV infection causes hemorrhagic symptoms and is associated with high mortality rates in humans, cats and cheetahs (Kato et al., 2016; Kobayashi et al., 2020; Matsuno et al., 2018; Matsuu et al., 2019) In Japan, the first SFTS patient was reported in Yamaguchi prefecture in autumn 2012 (Takahashi et al., 2013). Since then, SFTS patients have been reported in the western part of Japan; at present over 60 cases are reported per year with a case fatality ratio of 27% (Kobayashi et al., 2020).

SFTSV has a wide range of hosts, including humans and livestock, zoo, companion animals and wild animals. Epidemiological surveys have been reported from China, Korea and Japan, and have demonstrated anti-SFTSV antibody positivity in domesticated animals, including goats, sheep, cattle, dogs, chickens, and pigs, and wild animals including deer, wild boar, and rodents in endemic areas (Chen et al., 2019; Kimura et al., 2018; Kirino et al., 2022; Matsuu et al., 2021). The anti-SFTSV antibody positivity rate of animals tends to be higher in endemic areas where human SFTS patients have been reported (Chen et al., 2019; Kimura et al., 2018; Kirino et al., 2022; Matsuu et al., 2021), and seroprevalence in wild animals may be a superior indicator of the risk of SFTSV infection in humans. In this study, surveillance of SFTSV infection in Sika deer and wild boar in Yamaguchi prefecture was conducted using serum samples collected for 10-years from 2010, before the first human patient in Japan was identified.

3.3. Material and methods

3.3.1. Cells and viruses

Vero cells (Japanese Collection of Research Bioresources: JCRB9013) which is originated from African green monkey's kidney were used for viral amplification and neutralization test. Vero cells were cultured with Dulbecco's modified Eagle's medium (DMEM: Gibco, CA, USA) supplemented with 5% heat-inactivated fetal calf serum (FCS; Gibco), penicillin 100U/ml and streptomycin 100 μg /ml at 37 °C under 5% CO_2.

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 (Yu et al., 2011). HB29 was amplified in Vero cells and stored at -80°C.

3.3.2. Serum from Sika deer and wild boars

Serum samples for detection of antibodies were collected from 789 Sika deer (*Cervus nippon*) and 517 wild boars (*Sus scrofa*) in Yamaguchi Prefecture, Japan from January 2010 to February 2020. To detect SFTSV RNA, RT-PCR was performed using sera which were collected from 229 Sika deer and 116 wild boars captured during January 2019 and January 2022 in Yamaguchi prefecture. A part of sera used for detection of SFTSV RNA were also analyzed by FRNT50. The animals were captured by hunters as countermeasures under the official population control program. And a bio-information (body weight and sex) and capture date were recorded. All collected serum samples were stored at -20°C until use.

3.3.3. 50% focus-reduction neutralization test ($FRNT_{50}$)

To detect antibody from sera, a VN test was performed by a 50% of focus-

reduction neutralization test (FRNT50) using the SFTSV HB29 strain (Yu et al., 2011). Before the VN test, complement in sera was inactivated at 56℃ for 30 min. FRNT50 using Vero cells was carried with a final 10-fold dilution of sera. according to previous reports (Park et al., 2019) Briefly, heat-inactivated serum which is diluted in 5 fold-times by DMEM with 2% FCS and antibiotics were mixed with 2,000 focus-forming units/ml of SFTSV in equal volume and incubated at 37℃ for 60 min. 100ul of the mixture were applied on cultured Vero cells in a 12 well plate (SUMITOMO BAKELITE) and incubated at 37℃ for 60 min. Then after washing of cells by DMEM, cells were culture with overlay medium which is DMEM containing 2% FBS and 1% methylcellulose for a week. Cultured cells were fixed by 10% of buffered formalin and permeabilized by 0.1% of Triton-X100 (Sigma-Aldrich) in PBS. foci were stained by rabbit serum against SFTSV-N protein and protein A and G conjugated with HRP (Thermo Fisher Science) and DAB (FUJIFILM). Positive serum showed more than 50% of the number of focus-reduction compared with non-serum well.

3.3.4. Gene detection of SFTSV from serum

RNA was extracted from 140μL serum using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacture instructions.

RT-PCR was performed using a One-step RT-PCR kit (QIAGEN) with two primers targeting nucleocapsid protein gene of SFTSV (S2-200: 5’-GACACAAAGTTCATCATTGTCTTTGCCCT-3’, S2-360: 5’-TGCTGCAGCACATGTCCAAGTGG-3’) (Park et al., 2019). 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 bands were purified by MinElute Gel Extraction kit (QIAGEN).

3.3.5. Sequencing and phylogenetic analysis

The nucleotide sequence was determined from positive PCR bands using BigDye Terminator v.3.1 technology (FASMAC, Kanagawa, Japan). The obtained sequence was aligned with other SFTSV genome and construct phylogenetic tree to classify the genotype (Park et al., 2019).

3.4. Results

3.4.1. Seroprevalence of SFTSV infection in Sika deer and wild boars

The results of $FRNT_{50}$ showed that 510 of 789 Sika deer (64.6%) and 199 of 517 wild boars (38.5%) possessed VN antibodies against SFTSV (Table 1). Sika deer and wild boars showed antibody prevalence of 46.7% and 11.4%, respectively in 2010, which was 2 years before the first SFTS patients were identified in Yamaguchi prefecture. The antibody positivity rate in Sika deer gradually increased with time. From 2010 to 2013, the positivity rate in Sika deer was 42.3% (92/217), further increasing to 81.0% (324/400) from 2015 to 2020. Similarly, the anti-SFTSV antibody positivity rate in wild boars increased after 2015 (Figure 1). These results indicate that the virus is circulating more in the field environment after 2015, which may increase the risk of SFTSV transmission to humans.

In the seroprevalence, there was no significant difference in the seropositivity between female and male Sika deer or wild boars (Table 1). On the other hand, the prevalence of anti-SFTSV antibodies increased in a body weight-dependent manner in both Sika deer and wild boar populations (Table 1). The rates of anti-SFTSV antibody positivity in Sika deer of less than 30 kg, 30-59 kg, and 60 kg or more were 44.5%, 64.2%, and 73.3%, respectively. Similarly, rates of anti-SFTSV antibody positivity in wild boars of less than 30 kg, 30-59 kg, and 60 kg or more were 28.2%, 29.1% and 55.5%, respectively. These results indicated that heavier animals (i.e., older animals) have more opportunities to be infected with SFTSV. The similar result was reported in raccoons in Wakayama prefecture.

3.4.2. Detection of SFTSV genes from Sika deer and wild boars

To detect SFTSV RNA in Sika deer and wild boars, RT-PCR was performed using serum samples. Sera were collected from 229 Sika deer and 116 wild boars captured during January 2019 and January 2022 in Yamaguchi prefecture. As a result, SFTSV RNA was detected from 1 of 229 Sika deer (0.4%) and none of 116 wild boars (0%). The SFTSV-positive Sika deer was a male of 40 kg that was captured in January 2019 and possessed anti-SFTSV antibodies. The sequence of the amplified fragment (201 bp) was determined and deposited in the DNA Data Bank of Japan (DDBJ: accession number: LC709266). The SFTSV sequence obtained from the deer was phylogenetically classified into Japanese genotype, J1, which was also detected from human patients in Yamaguchi (Takahashi et al., 2013; Yoshikawa et al., 2015).

3.5. Discussion

To detect anti-SFTSV antibody from many animal species, we established an enzyme-linked immunosorbent assay (ELISA) using Protein A/G as a second antibody. In raccoons, the specificity and sensitivity of the ELISA were both 100%. On the other hand, the sensitivity and specificity of the ELISA for Sika deer and wild boars were low (our unpublished data). Therefore, we performed the VN test for the surveillance of SFTSV infection among Sika deer and wild boars.

In this study, high rates of seropositivity were found in both Sika deer and wild boars captured over a 10-year period. Seroprevalence in Yamaguchi prefecture was already high before 2012, and further increased after 2015. These results indicate that the risk of infection would have increased in humans and animals in this area from 2013 to 2015. The reason why the positive rate of SFTS virus infection increased sharply between 2013 and 2015 remains unknown. We need further examination and analysis for identifying the factor of increase of SFTS virus infection. In Yamaguchi prefecture, several SFTS patients have been reported each year since 2013 (Takahashi et al., 2013) and some cases of cats with SFTS have been reported since 2017 (our unpublished data). Sika deer may be a superior sentinel to assess the regional spread of SFTSV and the risk of infection to humans in endemic areas.

SFTSV RNA was detected only from one serum sample of a Sika deer. However, the kinetics of SFTSV in Sika deer remain poorly characterized. Further studies are required to understand the role of Sika deer in the transmission cycle of SFTSV. SFTSV RNA was not detected from wild boars and wild boars possessed lower positive ratio of anti-SFTSV antibody than Sika deer. In China, pigs were less infected with SFTSV than cattle and goats (Chen et al., 2013), suggesting that pigs and wild boars might be less

sensitive to SFTSV infection than the other animals belonging to the order *Artiodactyla*.

In conclusion, SFTSV infection had spread among wild animals in Yamaguchi prefecture before 2010 and recently expanded, resulting in annual reports of several cases in human patients in this region. Surveillance of SFTSV infection among wild animals, especially Sika deer, is useful for assessing the risk of SFTSV infection in humans.

3.6. Legend for figure

Figure 1. Seroprevalence of anti-SFTSV antibodies in Sika deer (black bar) and wild boar (white bar) by year. The arrow shows the year of the first report on human patient in Yamaguchi.

3.7. Figure and table

Fig.1

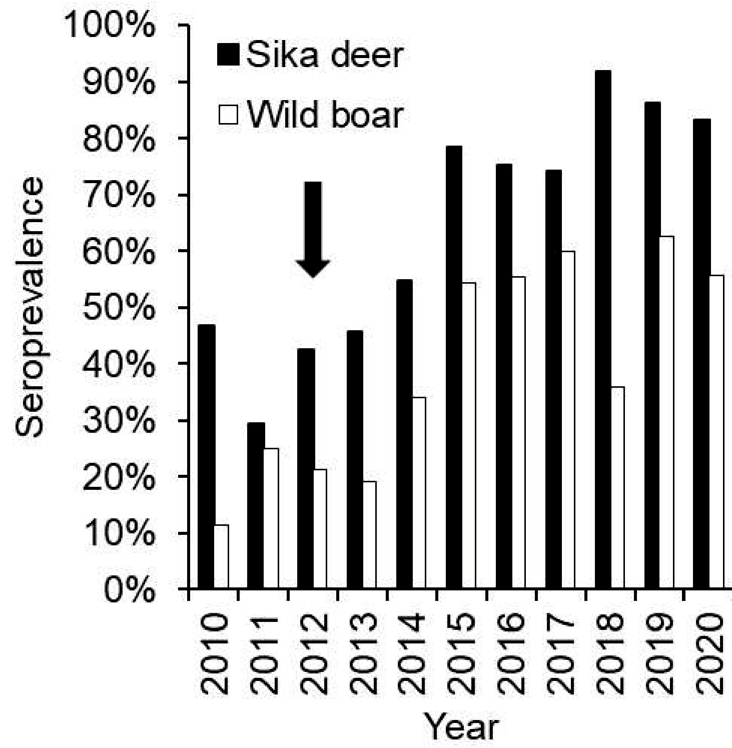

Table 1. Prevalence of anti-SFTSV antibody in Sika deer and wild boar in Yamaguchi by year, sex and body weight

		Sika deer			Wild boar		
		Number of examined animals	Number of positive animals	Percentage of positive animals	Number of examined animals	Number of positive animals	Percentage of positive animals
Year	2010	45	21	46.7%	44	5	11.4%
	2011	34	10	29.4%	24	6	25.0%
	2012	59	25	42.4%	42	9	21.4%
	2013	79	36	45.6%	47	9	19.1%
	2014	172	94	54.7%	100	34	34.0%
	2015	79	62	78.5%	59	32	54.2%
	2016	77	58	75.3%	85	47	55.3%
	2017	89	66	74.2%	30	18	60.0%
	2018	84	77	91.7%	53	19	35.8%
	2019	65	56	86.2%	24	15	62.5%
	2020	6	5	83.3%	9	5	55.6%
Sex	Male	310	195	62.9%	278	103	37.1%
	Female	457	300	65.6%	220	91	41.4%
	No data	22	15	68.2%	19	5	26.3%
Body weight	-29kg	119	53	44.5%	142	40	28.2%
	30kg-59kg	419	269	64.2%	172	50	29.1%
	60kg-	146	107	73.3%	137	76	55.5%
	No data	105	81	77.1%	66	33	50.0%
Total		789	510	64.6%	517	199	38.5%

4.General conclusion

SFTSV causes tick-borne zoonosis with high fatality rate in human, cats and cheetah. SFTSV are maintained in ticks or between ticks and animals in ecosystem. Therefore, understanding the roles of wild animals in transmission cycle of SFTSV contribute to know the risk of infection with SFTSV in nature.

In this study, I analysis SFTSV infection in wild animals in endemic area.

In CHAPTER 1, enzyme-linked immunosorbent assay (ELISA) system was developed and success to detect anti-SFTSV antibody in raccoon with high sensitivity and specificity. The serological analysis among raccoon in Wakayama prefecture during 2010 – 2019 revealed endemic of SFTSV among raccoon in that area. And SFTSV RNA were detected from 2.4% of raccoon's serum. The SFTS patients in that area have been reported since endemic among raccoon. The raccoon is a useful sentinel for assessing the risk of SFTSV infection in humans. Raccoons with viremia might be an important reservoir for SFTSV in nature.

In CHAPTER2, retrospective serological surveillance of SFTSV infection in Sika deer and wild boars in Yamaguchi prefecture, which the first SFTS patient was reported in Japan were performed using viral-neutralization test.

In 2010, which was 2 years before the first SFTS patients were identified, seroprevalence among Sika deer and wild boars was already high. Additionally, higher antibody prevalence was observed in Sika deer than wild boar. Importantly, the seroprevalence further increased after 2015. These results indicate that the risk of infection

would have increased in humans and animals in this area from 2013 to 2015. SFTSV infection had spread among wild animals in Yamaguchi prefecture before 2010 and recently expanded, resulting in annual reports of several cases in human patients in this region. Surveillance of SFTSV infection among wild animals, especially Sika deer, is available for analysis the risk of SFTSV infection in humans.

In this study, the infection of SFTSV in wild animals in endemic areas was analyzed. In Chapter1, the endemic of SFTSV in raccoons are correlated with the occurrence of SFTS patients in the area. The surveillance among raccoons is useful for risk analysis of SFTSV infection to human. Furthermore, raccoons might be one of the most important reservoir of SFTSV in this region. In Chapter2, serosurveillance of SFTSV among Sika deer and wild boar also contribute to assess the risk of SFTSV infection in endemic area. In Yamaguchi prefecture, which first patient was reported, SFTSV had been spreading among wild animals before discovery of the first SFTS patient and the risks further increased recently.

These findings indicated that raccoons, Sika deer and wild boars play important roles in virus transmission cycle of SFTSV in nature and surveillance of SFTSV infection among wildlife must be useful for the risk assessment of SFTSV in each area. Further study on SFTSV infection among other wild animals will be required to prevent SFTSV infection among wild animals and to reduce the risk of SFTSV infection in human.

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6. Reference

Ando T, Nabeshima T, Inoue S, Tun MMN, Obata M, Hu W, Shimoda H, Kurihara S, Izumikawa K, Morita K, Hayasaka D. 2021. Severe Fever with Thrombocytopenia Syndrome in Cats and Its Prevalence among Veterinarian Staff Members in Nagasaki, Japan. Viruses. 13:1142.

Casel MA, Park SJ, Choi YK. 2021. Severe fever with thrombocytopenia syndrome virus: emerging novel phlebovirus and their control strategy. Exp. Mol. Med. 53:713-722.

Chaudhary V, Zhang S, Yuen KS, Li C, Lui PY, Fung SY, Wang PH, Chan CP, Li D, Kok KH, Liang M, Jin DY. 2015. Suppression of type I and type III IFN signalling by NSs protein of severe fever with thrombocytopenia syndrome virus through inhibition of STAT1 phosphorylation and activation. J Gen Virol. 96:3204-3211.

Chen C, Li P, Li KF, Wang HL, Dai YX, Cheng X, Yan JB. 2019. Animals as amplification hosts in the spread of severe fever with thrombocytopenia syndrome virus: A systematic review and meta-analysis. Int. J. Infect. Dis. 79:77-84.

Chen XP, Cong ML, Li MH, Kang YJ, Feng YM, Plyusnin A, Xu J, Zhang YZ. 2012. Infection and pathogenesis of Huaiyangshan virus (a novel tick-borne bunyavirus) in laboratory rodents. J Gen Virol. 93:1288-1293.

Cui F, Cao HX, Wang L, Zhang SF, Ding SJ, Yu XJ, Yu H. 2013. Clinical and epidemiological study on severe fever with thrombocytopenia syndrome in Yiyuan County, Shandong Province, China. Am J Trop Med Hyg. 88:510-2.

Ding S, Yin H, Xu X, Liu G, Jiang S, Wang W, Han X, Liu J, Niu G, Zhang X, Yu XJ, Wang X. 2014. A cross-sectional survey of severe fever with thrombocytopenia syndrome virus infection of domestic animals in Laizhou City, Shandong Province, China. Jpn J Infect Dis. 67:1-4.

Dong F, Li D, Wen D, Li S, Zhao C, Qi Y, Jangra RK, Wu C, Xia D, Zhang X, Deng F,

Chandran K, Zou Z, Yuan F, Zheng A. 2019. Single dose of a rVSV-based vaccine elicits complete protection against severe fever with thrombocytopenia syndrome virus. NPJ Vaccines. 4:5.

Fukuma A, Fukushi S, Yoshikawa T, Tani H, Taniguchi S, Kurosu T, Egawa K, Suda Y, Singh H, Nomachi T, Gokuden M, Ando K, Kida K, Kan M, Kato N, Yoshikawa A, Kitamoto H, Sato Y, Suzuki T, Hasegawa H, Morikawa S, Shimojima M, Saijo M. 2016. Severe fever with thrombocytopenia syndrome virus antigen detection using monoclonal antibodies to the nucleocapsid protein. PLoS Negl. Trop. Dis. 10:e0004595.

Furuta Y, Komeno T, Nakamura T. 2017. Favipiravir (T-705), a broad spectrum inhibitor of viral RNA polymerase. Proc Jpn Acad Ser B Phys Biol Sci. 93:449-463.

Gowen BB, Westover JB, Miao J, Van Wettere AJ, Rigas JD, Hickerson BT, Jung KH, Li R, Conrad BL, Nielson S, Furuta Y, Wang Z. 2017 Modeling Severe Fever with Thrombocytopenia Syndrome Virus Infection in Golden Syrian Hamsters: Importance of STAT2 in Preventing Disease and Effective Treatment with Favipiravir. J Virol. 91:e01942-16.

Hashimoto T, Yahiro T, Yamada K, Kimitsuki K, Okuyama MW, Honda A, Kato M, Narimatsu H, Hiramatsu K, Nishizono A. 2022. Distribution of Severe Fever with Thrombocytopenia Syndrome Virus and Antiviral Antibodies in Wild and Domestic Animals in Oita Prefecture, Japan. Am J Trop Med Hyg. tpmd211130.

Hayasaka D, Fuxun Y, Yoshikawa A, Posadas-Herrera G, Shimada S, Tun MM, Agoh M, Morita K. 2016. Seroepidemiological evidence of severe fever with thrombocytopenia syndrome virus infections in wild boars in Nagasaki, Japan. Trop Med Health. 44:6.

Hofmann H, Li X, Zhang X, Liu W, Kühl A, Kaup F, Soldan SS, González-Scarano F, Weber

F, He Y, Pöhlmann S. 2013 Severe fever with thrombocytopenia virus glycoproteins are targeted by neutralizing antibodies and can use DC-SIGN as a receptor for pH-dependent entry into human and animal cell lines. J Virol. 87:4384-94.

Ikemori R, Aoyama I, Sasaki T, Takabayashi H, Morisada K, Kinoshita M, Ikuta K, Yumisashi T, Motomura K. 2021. Two Different Strains of Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV) in North and South Osaka by Phylogenetic Analysis of Evolutionary Lineage: Evidence for Independent SFTSV Transmission. Viruses. 13:177.

Jiao Y, Zeng X, Guo X, Qi X, Zhang X, Shi Z, Zhou M, Bao C, Zhang W, Xu Y, Wang H. 2012. Preparation and evaluation of recombinant severe fever with thrombocytopenia syndrome virus nucleocapsid protein for detection of total antibodies in human and animal sera by double-antigen sandwich enzyme-linked immunosorbent assay. J Clin Microbiol. 50:372-377.

Jin C, Jiang H, Liang M, Han Y, Gu W, Zhang F, Zhu H, Wu W, Chen T, Li C, Zhang W, Zhang Q, Qu J, Wei Q, Qin C, Li D. 2015. SFTS virus infection in nonhuman primates. J Infect Dis. 211:915-925.

Jin C, Liang M, Ning J, Gu W, Jiang H, Wu W, Zhang F, Li C, Zhang Q, Zhu H, Chen T, Han Y, Zhang W, Zhang S, Wang Q, Sun L, Liu Q, Li J, Wang T, Wei Q, Wang S, Deng Y, Qin C, Li D. 2012. Pathogenesis of emerging severe fever with thrombocytopenia syndrome virus in C57/BL6 mouse model. Proc Natl Acad Sci U S A. 109:10053-8.

Kanda Y. 2013. Investigation of the freely available easy-to-use software “EZR” for medical statistics. Bone Marrow Transplant. 48:452–458.

Kang JG, Cho YK, Jo YS, Chae JB, Oh SS, Kim KH, Ko MK, Yi J, Choi KS, Yu DH, Kim HC, Park J, Park BK, Choi CY, Jung YH, Chae JS. 2018. Prevalence of severe fever

with thrombocytopenia syndrome virus in black goats (Capra hircus coreanae) in the Republic of Korea. Ticks Tick Borne Dis. 9:1153-1157.

Kang JG, Jeon K, Choi H, Kim Y, Kim HI, Ro HJ, Seo YB, Shin J, Chung J, Jeon YK, Kim YS, Lee KH, Cho NH. 2020. Vaccination with single plasmid DNA encoding IL-12 and antigens of severe fever with thrombocytopenia syndrome virus elicits complete protection in IFNAR knockout mice. PLoS Negl Trop Dis. 14:e0007813.

Kato H, Yamagishi T, Shimada T, Matsui T, Shimojima M, Saijo M, Oishi K, Abe M, Wada M, Umekita K, Kamekou M, Tanioka D, Sanada I, Kuwai T, Tanaka Y, Shigetou K, Homma Y, Yamamoto C, Yamauchi M, Hayashi S, Watanabe S, Kitao A, Takatsu H, Nakanishi Y, Koguro K, Watanabe M, Uehara N, Kaneko M, Yamanaka A, Murakami Y, Konishi T, Sakamoto A, Harada M, Yamamoto K, Hayashi T, Kondo N, Suemori K, Ogawa T, Nakazawa R, Yamamoto Y, Miyahara M, Senba T, Maruhashi T, Fukushi S, Tani H, Yoshikawa T, Morikawa S. 2016. Epidemiological and clinical features of severe fever with thrombocytopenia syndrome in Japan, 2013–2014. PLoS One 11:e0165207.

Kida K, Matsuoka Y, Shimoda T, Matsuoka H, Yamada H, Saito T, Imataki O, Kadowaki N, Noguchi K, Maeda K, Mochizuki Y, Kishimoto T. 2019. A Case of Cat-to-Human Transmission of Severe Fever with Thrombocytopenia Syndrome Virus. Jpn J Infect Dis. 72:356-358

Kim KH, Yi J, Kim G, Choi SJ, Jun KI, Kim NH, Choe PG, Kim NJ, Lee JK, Oh MD. 2013. Severe fever with thrombocytopenia syndrome, South Korea, 2012. Emerg Infect Dis. 19:1892-4.

Kimura T, Fukuma A, Shimojima M, Yamashita Y, Mizota F, Yamashita M, Otsuka Y, Kan M, Fukushi S, Tani H, Taniguchi S, Ogata M, Kurosu T, Morikawa S, Saijo M,

Shinomiya H. 2018. Seroprevalence of severe fever with thrombocytopenia syndrome (SFTS) virus antibodies in humans and animals in Ehime prefecture, Japan, an endemic region of SFTS. J Infect Chemother. 24:802-806.

Kirino Y, Yamamoto S, Nomachi T, Mai TN, Sato Y, Sudaryatma PE, Norimine J, Fujii Y, Ando S, Okabayashi T. 2022. Serological and molecular survey of tick-borne zoonotic pathogens including severe fever with thrombocytopenia syndrome virus in wild boars in Miyazaki Prefecture, Japan. Vet Med Sci. 8:877-885.

Kirino Y, Yamanaka A, Ishijima K, Tatemoto K, Maeda K, Okabayashi T. 2022. Retrospective study on the possibility of an SFTS outbreak associated with undiagnosed febrile illness in veterinary professionals and a family with sick dogs in 2003. J Infect Chemother. 28:753-756.

Kobayashi Y, Kato H, Yamagishi T, Shimada T, Matsui T, Yoshikawa T, Kurosu T, Shimojima M, Morikawa S, Hasegawa H, Saijo M, Oishi K. 2020. Severe fever with thrombocytopenia syndrome, Japan, 2013-2017. Emerg. Infect. Dis. 26:692-699.

Kuba Y, Kyan H, Azama Y, Fukuchi Y, Park ES, Kakita T, Oyama M, Maeshiro N, Miyahira M, Nidaira M, Maeda K, Morikawa S, Taniguchi K. 2021. Seroepidemiological study of severe fever with thrombocytopenia syndrome in animals and humans in Okinawa, Japan. Ticks Tick Borne Dis. 12:101821.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33:1870-1874.

Kurihara S, Satoh A, Yu F, Hayasaka D, Shimojima M, Tashiro M, Saijo T, Takazono T, Imamura Y, Miyazaki T, Tsukamoto M, Yanagihara K, Mukae H, Saijo M, Morita K, Kohno S, Izumikawa K. 2016. The world first two cases of severe fever with thrombocytopenia syndrome: An epidemiological study in Nagasaki, Japan. J Infect

Chemother. 22:461-5.

Kwak JE, Kim YI, Park SJ, Yu MA, Kwon HI, Eo S, Kim TS, Seok J, Choi WS, Jeong JH, Lee H, Cho Y, Kwon JA, Jeong M, Maslow JN, Kim YE, Jeon H, Kim KK, Shin EC, Song MS, Jung JU, Choi YK, Park SH. 2019. Development of a SFTSV DNA vaccine that confers complete protection against lethal infection in ferrets. Nat Commun. 10:3836.

Lee HS, Kim J, Son K, Kim Y, Hwang J, Jeong H, Ahn TY, Jheong WH. 2020. Phylogenetic analysis of severe fever with thrombocytopenia syndrome virus in Korean water deer (Hydropotes inermis argyropus) in the Republic of Korea. Ticks Tick Borne Dis. 11:101331.

Lee J, Moon K, Kim M, Lee WG, Lee HI, Park JK, Kim YH. 2021. Seasonal distribution of Haemaphysalis longicornis (Acari: Ixodidae) and detection of SFTS virus in Gyeongbuk Province, Republic of Korea, 2018. Acta Trop. 221:106012.

Lee SH, Kim HJ, Byun JW, Lee MJ, Kim NH, Kim DH, Kang HE, Nam HM. 2017. Molecular detection and phylogenetic analysis of severe fever with thrombocytopenia syndrome virus in shelter dogs and cats in the Republic of Korea. Ticks Tick Borne Dis. 8:626-630.

Li H, Jiang XM, Cui N, Yuan C, Zhang SF, Lu QB, Yang ZD, Xin QL, Song YB, Zhang XA, Liu HZ, Du J, Fan XJ, Yuan L, Yuan YM, Wang Z, Wang J, Zhang L, Zhang DN, Wang ZB, Dai K, Bai JY, Hao ZN, Fan H, Fang LQ, Xiao G, Yang Y, Peng K, Wang HQ, Li JX, Zhang LK, Liu W. 2021. Clinical effect and antiviral mechanism of T-705 in treating severe fever with thrombocytopenia syndrome. Signal Transduct Target Ther. 6:145.

Li Z, Bao C, Hu J, Liu W, Wang X, Zhang L, Ji Z, Feng Z, Li L, Shen A, Liu X, Zhao H,

Tan W, Zhou J, Qi X, Zhu Y, Tang F, Cardona CJ, Xing Z. 2016. Ecology of the Tick-Borne Phlebovirus Causing Severe Fever with Thrombocytopenia Syndrome in an Endemic Area of China. PLoS Negl Trop Dis. 10:e0004574.

Li Z, Bao C, Hu J, Liu W, Wang X, Zhang L, Ji Z, Feng Z, Li L, Shen A, Liu X, Zhao H, Tan W, Zhou J, Qi X, Zhu Y, Tang F, Cardona CJ, Xing Z. 2016. Ecology of the tick-borne phlebovirus causing severe fever with thrombocytopenia syndrome in an endemic area of China. PLoS Negl. Trop. Dis. 10:e0004574.

Li Z, Hu J, Bao C, Li P, Qi X, Qin Y, Wang S, Tan Z, Zhu Y, Tang F, Zhou M. 2014. Seroprevalence of antibodies against SFTS virus infection in farmers and animals, Jiangsu, China. J Clin Virol. 60:185-9.

Liang S, Bao C, Zhou M, Hu J, Tang F, Guo X, Jiao Y, Zhang W, Luo P, Li L, Zhu K, Tan W, Lu Q, Ge H, Chen A. 2014. Seroprevalence and risk factors for severe fever with thrombocytopenia syndrome virus infection in Jiangsu Province, China, 2011. Am J Trop Med Hyg. 90:256-9.

Lin TL, Ou SC, Maeda K, Shimoda H, Chan JP, Tu WC, Hsu WL, Chou CC. 2020. The first discovery of severe fever with thrombocytopenia syndrome virus in Taiwan. Emerg Microbes Infect. 9:148-151.

Liu JW, Wen HL, Fang LZ, Zhang ZT, He ST, Xue ZF, Ma DQ, Zhang XS, Wang T, Yu H, Zhang Y, Zhao L, Yu XJ. 2014. Prevalence of SFTSV among Asian house shrews and rodents, China, January-August 2013. Emerg Infect Dis. 20:2126-8.

Liu R, Huang DD, Bai JY, Zhuang L, Lu QB, Zhang XA, Liu W, Wang JY, Cao WC. 2015. Immunization with recombinant SFTSV/NSs protein does not promote virus clearance in SFTSV-infected C57BL/6J mice. Viral Immunol. 28:113-22.

Liu Y, Li Q, Hu W, Wu J, Wang Y, Mei L, Walker DH, Ren J, Wang Y, Yu XJ. 2012. Person-

to-person transmission of severe fever with thrombocytopenia syndrome virus. Vector Borne Zoonotic Dis. 12:156-60.

Liu Y, Wu B, Paessler S, Walker DH, Tesh RB, Yu XJ. 2014. The pathogenesis of severe fever with thrombocytopenia syndrome virus infection in alpha/beta interferon knockout mice: insights into the pathologic mechanisms of a new viral hemorrhagic fever. J Virol. 88:1781-6.

Lokupathirage SMW, Tsuda Y, Ikegame K, Noda K, Muthusinghe DS, Kozawa F, Manzoor R, Shimizu K, Yoshimatsu K. 2021. Subcellular localization of nucleocapsid protein of SFTSV and its assembly into the ribonucleoprotein complex with L protein and viral RNA. Sci Rep. 11:22977.

Luo LM, Zhao L, Wen HL, Zhang ZT, Liu JW, Fang LZ, Xue ZF, Ma DQ, Zhang XS, Ding SJ, Lei XY, Yu XJ. 2015. Haemaphysalis longicornis Ticks as Reservoir and Vector of Severe Fever with Thrombocytopenia Syndrome Virus in China. Emerg Infect Dis. 21:1770-6.

Matsuno K, Nonoue N, Noda A, Kasajima N, Noguchi K, Takano A, Shimoda H, Orba Y, Muramatsu M, Sakoda Y, Takada A, Minami S, Une Y, Morikawa S, Maeda K. 2018. Fatal Tickborne Phlebovirus Infection in Captive Cheetahs, Japan. Emerg Infect Dis. 24:1726-1729.

Matsuno K, Orba Y, Maede-White K, Scott D, Feldmann F, Liang M, Ebihara H. 2017. Animal Models of Emerging Tick-Borne Phleboviruses: Determining Target Cells in a Lethal Model of SFTSV Infection. Front Microbiol. 8:104.

Matsuu A, Hamakubo E, Yabuki M. 2021. Seroprevalence of severe fever with thrombocytopenia syndrome virus in animals in Kagoshima Prefecture, Japan, and development of Gaussia luciferase immunoprecipitation system to detect specific IgG

antibodies. Ticks Tick Borne Dis. 12:101771.

Matsuu A, Momoi Y, Nishiguchi A, Noguchi K, Yabuki M, Hamakubo E, Take M, Maeda K. 2019. Natural severe fever with thrombocytopenia syndrome virus infection in domestic cats in Japan. Vet Microbiol. 236:108346.

Miao D, Liu MJ, Wang YX, Ren X, Lu QB, Zhao GP, Dai K, Li XL, Li H, Zhang XA, Shi WQ, Wang LP, Yang Y, Fang LQ, Liu W. 2021. Epidemiology and Ecology of Severe Fever With Thrombocytopenia Syndrome in China, 2010–2018. Clin Infect Dis. 73:e3851-e3858.

Miyauchi A, Sada KE, Yamamoto H, Iriyoshi H, Touyama Y, Hashimoto D, Nojima S, Yamanaka S, Ishijima K, Maeda K, Kawamura M. 2022. Suspected Transmission of Severe Fever with Thrombocytopenia Syndrome Virus from a Cat to a Veterinarian by a Single Contact: A Case Report. Viruses. 14:223.

Moriyama M, Igarashi M, Koshiba T, Irie T, Takada A, Ichinohe T. 2018. Two Conserved Amino Acids within the NSs of Severe Fever with Thrombocytopenia Syndrome Phlebovirus Are Essential for Anti-interferon Activity. J Virol. 92:e00706-18.

National Institute of Infectious Diseases (NIID). 2022. Infectious Diseases Weekly Report (IDWR). https://www.niid.go.jp/niid/en/idwr-e.html [accessed on May 24, 2022]

Ning YJ, Feng K, Min YQ, Cao WC, Wang M, Deng F, Hu Z, Wang H. 2015. Disruption of type I interferon signaling by the nonstructural protein of severe fever with thrombocytopenia syndrome virus via the hijacking of STAT2 and STAT1 into inclusion bodies. J Virol. 89:4227-36.

Niu G, Li J, Liang M, Jiang X, Jiang M, Yin H, Wang Z, Li C, Zhang Q, Jin C, Wang X, Ding S, Xing Z, Wang S, Bi Z, Li D. 2013. Severe fever with thrombocytopenia syndrome virus among domesticated animals, China. Emerg Infect Dis. 19:756-63.

Oh SS, Chae JB, Kang JG, Kim HC, Chong ST, Shin JH, Hur MS, Suh JH, Oh MD, Jeong SM, Shin NS, Choi KS, Chae JS. 2016. Detection of Severe Fever with Thrombocytopenia Syndrome Virus from Wild Animals and Ixodidae Ticks in the Republic of Korea. Vector Borne Zoonotic Dis. 16 :408-14.

Ohagi Y, Tamura S, Nakamoto C, Nakamoto H, Saijo M, Shimojima M, Nakano Y, Fujimoto T. 2014. Mild clinical course of severe fever with thrombocytopenia syndrome virus infection in an elderly Japanese patient. Case Rep. Infect. Dis. 2014:918135.

Okada A, Hotta A, Kimura M, Park ES, Morikawa S, Inoshima Y. 2021. A retrospective survey of the seroprevalence of severe fever with thrombocytopenia syndrome virus in wild animals in Japan. Vet Med Sci. 7:600-605.

Oshima H, Okumura H, Maeda K, Ishijima K, Yoshikawa T, Kurosu T, Fukushi S, Shimojima M, Saijo M. A 2022. Patient with Severe Fever with Thrombocytopenia Syndrome (SFTS) Infected from a Sick Dog with SFTS Virus Infection. Jpn J Infect Dis. (in press)

Park ES, Fujita O, Kimura M, Hotta A, Imaoka K, Shimojima M, Saijo M, Maeda K, Morikawa S. 2021. Diagnostic system for the detection of severe fever with thrombocytopenia syndrome virus RNA from suspected infected animals. PLoS One. 16:e0238671.

Park ES, Shimojima M, Nagata N, Ami Y, Yoshikawa T, Iwata-Yoshikawa N, Fukushi S, Watanabe S, Kurosu T, Kataoka M, Okutani A, Kimura M, Imaoka K, Hanaki K, Suzuki T, Hasegawa H, Saijo M, Maeda K, Morikawa S. 2019. Severe Fever with Thrombocytopenia Syndrome Phlebovirus causes lethal viral hemorrhagic fever in cats. Sci Rep. 9:11990.

Park SJ, Kim YI, Park A, Kwon HI, Kim EH, Si YJ, Song MS, Lee CH, Jung K, Shin WJ,

Zeng J, Choi Y, Jung JU, Choi YK. 2019. Ferret animal model of severe fever with thrombocytopenia syndrome phlebovirus for human lethal infection and pathogenesis. Nat Microbiol. 4:438-446.

Park SW, Han MG, Yun SM, Park C, Lee WJ, Ryou J. 2014. Severe fever with thrombocytopenia syndrome virus, South Korea, 2013. Emerg. Infect. Dis. 20:1880-1882.

Peng SH, Yang SL, Tang SE, Wang TC, Hsu TC, Su CL, Chen MY, Shimojima M, Yoshikawa T, Shu PY. 2020. Human Case of Severe Fever with Thrombocytopenia Syndrome Virus Infection, Taiwan, 2019. Emerg Infect Dis. 26:1612-1614.

Plegge T, Hofmann-Winkler H, Spiegel M, Pöhlmann S. 2016. Evidence that Processing of the Severe Fever with Thrombocytopenia Syndrome Virus Gn/Gc Polyprotein Is Critical for Viral Infectivity and Requires an Internal Gc Signal Peptide. PLoS One. 11:e0166013.

Rim JM, Han SW, Cho YK, Kang JG, Choi KS, Jeong H, Son K, Kim J, Choi Y, Kim WM, Cho NH, Chae JS. 2021. Survey of severe fever with thrombocytopenia syndrome virus in wild boar in the Republic of Korea. Ticks Tick Borne Dis. 12:101813.

Saijo M. 2020. Pathophysiology of severe fever with thrombocytopenia syndrome and development of specific antiviral therapy. J Infect Chemother. 24:773-781.

Sakai Y, Kuwabara Y, Ishijima K, Kagimoto S, Mura S, Tatemoto K, Kuwata R, Yonemitsu K, Minami S, Kuroda Y, Baba K, Okuda M, Shimoda H, Sakurai M, Morimoto M, Maeda K. 2021. Histopathological Characterization of Cases of Spontaneous Fatal Feline Severe Fever with Thrombocytopenia Syndrome, Japan. Emerg Infect Dis. 27:1068-1076.

Sano S, Fukushi S, Yamada S, Harada S, Kinoshita H, Sugimoto S, Yoshikawa T, Kurosu T,

Takamatsu Y, Shimojima M, Toda S, Hamada Y, Fujisawa N, Sugimoto T, Saijo M. 2021. Development of an RT-LAMP Assay for the Rapid Detection of SFTS Virus. Viruses. 13:693.

Sato Y, Mekata H, Sudaryatma PE, Kirino Y, Yamamoto S, Ando S, Sugimoto T, Okabayashi T. 2021. Isolation of Severe Fever with Thrombocytopenia Syndrome Virus from Various Tick Species in Area with Human Severe Fever with Thrombocytopenia Syndrome Cases. Vector Borne Zoonotic Dis. 21:378-384.

Sun Y, Liu MM, Luo LM, Zhao L, Wen HL, Zhang ZT, Liu JW, Xue ZF, Ma DQ, Ding SJ, Lei XY, Yu XJ. 2017. Seroprevalence of Severe Fever with Thrombocytopenia Syndrome Virus in Hedgehog from China. Vector Borne Zoonotic Dis. 17:347-350.

Suzuki J, Nishio Y, Kameo Y, Terada Y, Kuwata R, Shimoda H, Suzuki K, Maeda K. 2015. Canine distemper virus infection among wildlife before and after the epidemic. J. Vet. Med. Sci. 77:1457-1463.

Tabara K, Fujita H, Hirata A, Hayasaka D. 2016. Investigation of Severe Fever with Thrombocytopenia Syndrome Virus Antibody among Domestic Bovines Transported to Slaughterhouse in Shimane Prefecture, Japan. Jpn J Infect Dis. 69:445-7.

Takahashi T, Maeda K, Suzuki T, Ishido A, Shigeoka T, Tominaga T, Kamei T, Honda M, Ninomiya D, Sakai T, Senba T, Kaneyuki S, Sakaguchi S, Satoh A, Hosokawa T, Kawabe Y, Kurihara S, Izumikawa K, Kohno S, Azuma T, Suemori K, Yasukawa M, Mizutani T, Omatsu T, Katayama Y, Miyahara M, Ijuin M, Doi K, Okuda M, Umeki K, Saito T, Fukushima K, Nakajima K, Yoshikawa T, Tani H, Fukushi S, Fukuma A, Ogata M, Shimojima M, Nakajima N, Nagata N, Katano H, Fukumoto H, Sato Y, Hasegawa H, Yamagishi T, Oishi K, Kurane I, Morikawa S, Saijo M. 2014. The first identification and retrospective study of severe fever with thrombocytopenia syndrome in Japan. J.

Infect. Dis. 209:816-27.

Tani H, Fukuma A, Fukushi S, Taniguchi S, Yoshikawa T, Iwata-Yoshikawa N, Sato Y, Suzuki T, Nagata N, Hasegawa H, Kawai Y, Uda A, Morikawa S, Shimojima M, Watanabe H, Saijo M. 2016. Efficacy of T-705 (Favipiravir) in the Treatment of Infections with Lethal Severe Fever with Thrombocytopenia Syndrome Virus. mSphere. 1:e00061-15.

Tani H. 2014. Analyses of Entry Mechanisms of Novel Emerging Viruses Using Pseudotype VSV System. Trop Med Health. 42:71-82.

Tani H, Komeno T, Fukuma A, Fukushi S, Taniguchi S, Shimojima M, Uda A, Morikawa S, Nakajima N, Furuta Y, Saijo M. 2018. Therapeutic effects of favipiravir against severe fever with thrombocytopenia syndrome virus infection in a lethal mouse model: Dose-efficacy studies upon oral administration. PLoS One. 13:e0206416.

Taniguchi S, Fukuma A, Tani H, Fukushi S, Saijo M, Shimojima M. 2017. A neutralization assay with a severe fever with thrombocytopenia syndrome virus strain that makes plaques in inoculated cells. J. Virol. Methods. 244:4-10.

Tatemoto K, Ishijima K, Kuroda Y, Mendoza MV, Inoue Y, Park ES, Shimoda H, Sato Y, Suzuki T, Suzuki K, Morikawa S, Maeda K. 2022. Roles of raccoons in the transmission cycle of severe fever with thrombocytopenia syndrome virus. J. Vet. Med. Sci. (in press)

Ter Horst S, Conceição-Neto N, Neyts J, Rocha-Pereira J. 2019. Structural and functional similarities in bunyaviruses: Perspectives for pan-bunya antivirals. Rev Med Virol. 29:e2039.

Tian L, Yan L, Zheng W, Lei X, Fu Q, Xue X, Wang X, Xia X, Zheng X. 2021. A rabies virus vectored severe fever with thrombocytopenia syndrome (SFTS) bivalent

candidate vaccine confers protective immune responses in mice. Vet Microbiol. 257:109076.

Tran XC, Yun Y, Van An L, Kim SH, Thao NTP, Man PKC, Yoo JR, Heo ST, Cho NH, Lee KH. 2019. Endemic Severe Fever with Thrombocytopenia Syndrome, Vietnam. Emerg Infect Dis. 25:1029-1031.

Uchida L, Hayasaka D, Ngwe Tun MM, Morita K, Muramatsu Y, Hagiwara K. 2018. Survey of tick-borne zoonotic viruses in wild deer in Hokkaido, Japan. J Vet Med Sci. 80:985-988.

Wang GS, Wang JB, Tian FL, Zhang HJ, Yin FF, Xu C, Xu D, Huang YT, Yu XJ. 2017.Severe Fever with Thrombocytopenia Syndrome Virus Infection in Minks in China. Vector Borne Zoonotic Dis. 17:596-598.

Wang S, Li J, Niu G, Wang X, Ding S, Jiang X, Li C, Zhang Q, Liang M, Bi Z, Li D. 2015. SFTS virus in ticks in an endemic area of China. Am J Trop Med Hyg. 92:684-9.

Xing X, Guan X, Liu L, Zhan J, Jiang H, Liu L, Li G, Xiong J, Tan L, Xu J, Jiang Y, Yao X, Zhan F, Nie S. 2016. Natural Transmission Model for Severe Fever With Thrombocytopenia Syndrome Bunyavirus in Villages of Hubei Province, China. Medicine (Baltimore). 95:e2533.

Xing Z, Schefers J, Schwabenlander M, Jiao Y, Liang M, Qi X, Li C, Goyal S, Cardona CJ, Wu X, Zhang Z, Li D, Collins J, Murtaugh MP. 2013. Novel bunyavirus in domestic and captive farmed animals, Minnesota, USA. Emerg Infect Dis. 19:1487-9.

Yamanaka A, Kirino Y, Fujimoto S, Ueda N, Himeji D, Miura M, Sudaryatma PE, Sato Y, Tanaka H, Mekata H, Okabayashi T. 2020. Direct Transmission of Severe Fever with Thrombocytopenia Syndrome Virus from Domestic Cat to Veterinary Personnel. Emerg Infect Dis. 26:2994-2998.

Yoshikawa R, Sakabe S, Urata S, Yasuda J. 2019. Species-Specific Pathogenicity of Severe Fever with Thrombocytopenia Syndrome Virus Is Determined by Anti-STAT2 Activity of NSs. J Virol. 93:e02226-18.

Yoshikawa T, Shimojima M, Fukushi S, Tani H, Fukuma A, Taniguchi S, Singh H, Suda Y, Shirabe K, Toda S, Shimazu Y, Nomachi T, Gokuden M, Morimitsu T, Ando K, Yoshikawa A, Kan M, Uramoto M, Osako H, Kida K, Takimoto H, Kitamoto H, Terasoma F, Honda A, Maeda K, Takahashi T, Yamagishi T, Oishi K, Morikawa S, Saijo M. 2015. Phylogenetic and Geographic Relationships of Severe Fever With Thrombocytopenia Syndrome Virus in China, South Korea, and Japan. J Infect Dis. 212:889-98.

Yoshikawa T, Taniguchi S, Kato H, Iwata-Yoshikawa N, Tani H, Kurosu T, Fujii H, Omura N, Shibamura M, Watanabe S, Egawa K, Inagaki T, Sugimoto S, Phanthanawiboon S, Harada S, Yamada S, Fukushi S, Morikawa S, Nagata N, Shimojima M, Saijo M. 2021. A highly attenuated vaccinia virus strain LC16m8-based vaccine for severe fever with thrombocytopenia syndrome. PLoS Pathog. 17:e1008859.

Yoshikawa T. 2021. Vaccine Development for Severe Fever with Thrombocytopenia Syndrome. Viruses. 13:627.

Yu F, Du Y, Huang X, Ma H, Xu B, Adungo F, Hayasaka D, Buerano CC, Morita K. 2015. Application of recombinant severe fever with thrombocytopenia syndrome virus nucleocapsid protein for the detection of SFTSV-specific human IgG and IgM antibodies by indirect ELISA. Virol J. 12:117.

Yu KM, Park SJ, Yu MA, Kim YI, Choi Y, Jung JU, Brennan B, Choi YK. 2019. Cross-genotype protection of live-attenuated vaccine candidate for severe fever with thrombocytopenia syndrome virus in a ferret model. Proc Natl Acad Sci U S A.

116:26900–8.

Yu XJ, Liang MF, Zhang SY, Liu Y, Li JD, Sun YL, Zhang L, Zhang QF, Popov VL, Li C, Qu J, Li Q, Zhang YP, Hai R, Wu W, Wang Q, Zhan FX, Wang XJ, Kan B, Wang SW, Wan KL, Jing HQ, Lu JX, Yin WW, Zhou H, Guan XH, Liu JF, Bi ZQ, Liu GH, Ren J, Wang H, Zhao Z, Song JD, He JR, Wan T, Zhang JS, Fu XP, Sun LN, Dong XP, Feng ZJ, Yang WZ, Hong T, Zhang Y, Walker DH, Wang Y, Li DX. 2011. Fever with thrombocytopenia associated with a novel bunyavirus in China. N Engl J Med. 364:1523-32.

Yun MR, Ryou J, Choi W, Lee JY, Park SW, Kim DW. 2020. Genetic diversity and evolutionary history of Korean isolates of severe fever with thrombocytopenia syndrome virus from 2013-2016. Arch Virol. 165:2599-2603.

Yun SM, Lee YJ, Choi W, Kim HC, Chong ST, Chang KS, Coburn JM, Klein TA, Lee WJ. 2016. Molecular detection of severe fever with thrombocytopenia syndrome and tick-borne encephalitis viruses in ixodid ticks collected from vegetation, Republic of Korea, 2014. Ticks Tick Borne Dis. 7:970-978.

Yun SM, Park SJ, Kim YI, Park SW, Yu MA, Kwon HI, Kim EH, Yu KM, Jeong HW, Ryou J, Lee WJ, Jee Y, Lee JY, Choi YK. 2020. Genetic and pathogenic diversity of severe fever with thrombocytopenia syndrome virus (SFTSV) in South Korea. JCI Insight. 5:e129531.

Yun Y, Heo ST, Kim G, Hewson R, Kim H, Park D, Cho NH, Oh WS, Ryu SY, Kwon KT, Medlock JM, Lee KH. 2015. Phylogenetic Analysis of Severe Fever with Thrombocytopenia Syndrome Virus in South Korea and Migratory Bird Routes Between China, South Korea, and Japan. Am J Trop Med Hyg. 93:468-74.

Zhuang L, Sun Y, Cui XM, Tang F, Hu JG, Wang LY, Cui N, Yang ZD, Huang DD, Zhang

XA, Liu W, Cao WC. 2018. Transmission of Severe Fever with Thrombocytopenia Syndrome Virus by Haemaphysalis longicornis Ticks, China. Emerg Infect Dis. 24:868–71.

7. Abstract (in Japanese)

学位論文要旨

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Epidemiological studies on severe fever with thrombocytopenia syndrome virus among wild animals
重症熱性血小板減少症候群ウイルスの野生動物における疫学調査

重症熱性血小板減少症候群ウイルス（Sever fever with thrombocytopenia syndrome virus: SFTSV）はブニヤウイルス目フェヌイウイルス科バンダウイルス属に属するウイルスである。多くの動物種に感染し広い宿主域を持つマダニ媒介性の動物由来感染症であり、SFTSV に感染したヒト、ネコ、チーターは出血熱様症状を引き起こし高い致死率を示す。現在日本では毎年 100 例近くのヒト患者が報告されている。近年では SFTSV に感染したネコやイヌによる咬傷や分泌物を介したヒトの感染例が報告され公衆衛生上の問題となっている

自然界で SFTSV はマダニの発育ステージの中でウイルスが維持されるマダニサイクルと、感染した動物を介し、新たにマダニがウイルスを受け取る動物サイクルで維持されている。野生動物はマダニの吸血源となることで環境中のマダニを増やし、マダニにウイルスを供給することで感染マダニを増やす、という 2 点で、SFTSV の感染環の重要な役割を担っていると考えられる。しかし、野生動物の増幅動物としての役割やウイルスへの感受性などは未だ不明な点が多いのが現状である。

本研究は SFTS 患者の発生地域に生息する野生動物における SFTSV の感染状況を評価した 2 章より構成される。

第一章　アライグマにおける SFTSV の疫学研究

本章では SFTS 患者の報告地域である和歌山県におけるアライグマを対象に SFTSV の感染状況を評価した。大規模な血清疫学調査を実施するために、血清を用いた ELISA 法による抗体検出系を確立した。ウイルス中和試験と比較し ROC 解析を実施した結果、カットオフ値 OD≧0.564 で感度と特異度、共に 100%を示す ELISA 系の確立に成功した。

2007 年から 2019 年に捕獲された和歌山県内のアライグマを ELISA 法により調査した結果、4129 頭中 1334 頭（32.3%）が抗体を保有しており、その内、田辺地域で捕獲されたアライグマでは 2299 頭中 916 頭（32.9%）が抗体陽性を示した。田辺地域の抗体陽性率を年代別に比較した結果、2013 年 3 月まで 10%以下であった抗体陽性率が、その後上昇し、2015 年 4 月以降では 50%以上の高い抗体陽性率が示され、田辺地域のアライグマにおいて SFTSV の感染が拡大していたことが分かった。体重別の抗体陽性率では 2kg 未満で 66.3%であるのに対し、2-3kg で 38.5%、3-4kg で 30%と低く、幼獣のアライグマは移行抗体を保有していることが明らかとなった。血清を用いた SFTSV 遺伝子検出では全体で 2.4%の遺伝子陽性率を示した。捕獲月別では 1-3 月で 0.7%であるのに対し、4-6 月では 3.4%と高く、マダニが活発となる季節性を反映しているものと考えられた。SFTSV に感染したアライグマでは臓器や糞便中からも遺伝子が検出され、直腸のリンパ組織ではウイルス抗原も検出された。

和歌山県内では 2014 年に初めてのヒトにおける SFTS の症例が報告されており、アライグマにおける SFTSV の感染拡大と一致していた。即ち、アライグマを用いた SFTSV の流行調査がヒトにおける SFSTV の感染リスクの評価に有用であることが示された。

第二章　シカ及びイノシシの血清学的疫学調査による SFTSV 感染リスクの評価

2012 年秋に不明熱で死亡した山口県在住の患者から SFTSV が分離され、この症例が日本国内で、はじめての SFTS 症例として報告された。本章では、国内初の症例が報告された山口県において、患者が報告された 2012 年以前の 2010 年から 2020 年に採取されたシカ及びイノシシの血清を用いたウイルス中和試験

による血清疫学調査を実施した。その結果、789 頭中 510 頭（64.6%）のシカ、及び 517 頭中 199 頭（38.5%）のイノシシが中和抗体を保有しており、シカで高い抗体陽性率が示された。山口県内でヒト症例が報告される以前の 2010 年には、すでに 46.7%のシカ、11.4%のイノシシが抗体を保有しており、患者が報告される以前より SFTSV が分布していることが確認された。2010 年から 2013 年のシカの抗体陽性率は 42.3%であったが、2015 年から 2020 年では 81.0%と上昇していた。同じ傾向がイノシシについても認められ、18.5%から 52.3%に上昇しており、患者が発生した 2012 年以降においても、この地域における SFTSV に感染するリスクが上昇していることが推察された。血清を用いた遺伝子検査では 229 頭中 1 頭（0.4%）のシカがウイルス遺伝子を保有しており、116 頭のイノシシから SFTSV 遺伝子は検出されなかった。

以上のことより、国内初の SFTS 症例が報告された山口県では、2012 年以前から SFTSV が分布しており、症例が報告された 2012 年以降において SFTSV の感染リスクが更に上昇している可能性が示された。又、イノシシと比較しシカで高い抗体陽性率が示されたことから、特に、シカは SFTSV の感染における歩哨動物として有用であることが示された。

本研究は、アライグマ、シカ及びイノシシといった野生動物における SFTSV の感染状況を把握することで、地域におけるヒトへの感染リスクの評価や自然環境における SFTSV の流行動態の解明に成功した。現在、日本では SFTSV の分布地域の拡大が問題となっている。野生動物を対象とした疫学研究は地域の SFTSV 感染リスク評価に貢献できる有用な疫学手法であると考えられた。