

**Development of RT-LAMP assay for SFTS and Susceptibility of Mammalian Cell Lines to
SFTSV Infection**

SFTS の RT-LAMP 法開発と SFTS ウイルス感染における哺乳類細胞株の感受性

**Joint Graduate School of Veterinary Medicine
Yamaguchi University**

Marla Anggita

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1. ABSTRACT

Severe Fever with Thrombocytopenia Syndrome (SFTS) is an emerging tick-borne viral disease caused by SFTS virus (SFTSV), a phlebovirus within the family *Phenuiviridae*, has become major public health concern across East Asia including Japan, South Korea, and Taiwan, showing mortality rates of up to 30%. SFTSV demonstrates broad host range including humans, domestic animals, livestock, and wildlife. The risk of infection is highest among elderly individuals, and people engaged in agricultural and animal handling, such as farmers or veterinarians. The virus primarily transmitted by the bite of tick *Haemaphysalis longicornis*, with the recent human-to-human transmission has been documented. Clinical manifestations in humans include high fever, thrombocytopenia, leukopenia, gastrointestinal symptoms, and multi-organ dysfunction. SFTS diagnosis relies on laboratory confirmation through various methods, including virus isolation, RNA detection, and antibody testing. In areas with limited laboratory facilities, rapid diagnostic methods are being developed to cover the diagnostic capacity. Currently, no vaccine available for SFTSV prevention and only favipiravir was approved as the antiviral treatment for SFTSV (Li et al. 2021; Saijo et al. 2025). This critical gap, combined with SFTSV expanding geographic range and high mortality rate, underscores the urgent need for rapid diagnostic tools and comprehensive understanding of viral cellular tropism. This study focuses on two objectives: first, to develop a simplified, easy to perform diagnostic tool suitable for resource-limited settings, and second, to systematically evaluate SFTSV infectivity across diverse mammalian cell lines including original cells from wildlife to identify optimal cell culture systems for virological research.

The first chapter was conducted to address the diagnostic gaps in SFTS-endemic regions lacking with diagnostic infrastructure. We developed a ready-to-use dried reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay to enable detection

from clinical samples directly. This diagnostic tool employs a single-tube format that allows direct analysis of serum or plasma specimens without RNA extraction, simplifying the workflow compared to conventional molecular diagnostic methods. The assay could be performed in resource-limited areas in a short time, and the result could be easily observed under simple LED light illumination. Our validation study demonstrates successful detection of natural SFTSV infection in feline and SFTSV experimental mice samples, confirming the applicability for veterinary and potentially human diagnostic applications. While the SFTSV RT-LAMP assay demonstrates lower sensitivity compared to conventional RT-PCR, it offers significant advantages in speed, cost-effectiveness, technical simplicity, and applicability for point-of-care testing (POCT). These characteristics make it well-suited as an accessible tool for early SFTSV detection and routine animal surveillance in remote, resource-limited areas.

The second chapter was evaluating the SFTSV infectivity across multiple mammalian cell lines representing diverse species origins, including the original cell lines from tiger, wild deer, and hedgehog alongside commercial cell lines from feline, porcine, primate, human, and hamster origin. The comprehensive analysis assessed cytopathic effect (CPE) formation and viral growth kinetics following SFTSV infection across this diverse cellular panel. The results reveal the heterogeneity in cellular responses, with the feline-derived cell lines and porcine cells exhibiting CPE, whereas the other cells demonstrated absent or minimal CPE despite viral infection. Quantitative analysis of viral titers revealed that feline and primate (Vero) derived cells support the replication of infective SFTSV compared to the other cells, while hedgehog cells exhibited restricted viral replication. Notably, original cell from tiger demonstrated exceptional utility for SFTSV quantification by clear plaque formation, a characteristic that

absent in most conventional cell culture system for SFTSV due to limited CPE, establishing tiger cells as a valuable tool for plaque forming assays.

These studies aim to provide a diagnostic tool for early detection of SFTSV and systematic characterization of cellular infectivity pattern to SFTSV infection, thereby contributing to strengthened public health systems for preparedness and early response to this emerging threat and providing fundamental insights into SFTSV pathogenicity.

2. GENERAL INTRODUCTION

Severe fever with thrombocytopenia syndrome (SFTS) virus, a tick-borne pathogen from the family *Phenuiviridae*, has emerged as a major public health threat in East Asia. Since its discovery in 2011, SFTS has been reported in China, Japan, South Korea, Vietnam, and Taiwan, with thousands of cases documented and case fatality rates ranging from 12% to 30% depending on age, comorbidities, and access to medical care. The present study addresses critical needs in SFTSV research through two complementary approaches: characterizing differential cellular susceptibility across mammalian cell lines to establish optimal infection models of SFTSV and developing a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for rapid, equipment-free point-of-care diagnosis of SFTSV in resource limited areas.

2.1. Severe Fever with Thrombocytopenia Syndrome

Severe Fever with Thrombocytopenia Syndrome (SFTS) is an emerging tick-borne viral disease caused by SFTS virus (SFTSV), officially renamed *Bandavirus dabieense* in the 2024 International Committee on Taxonomy of Viruses (ICTV), belonging to the family *Phenuiviridae* within the class *Bunyaviricete*, order *Bunyavirales*. SFTSV is an enveloped, single-stranded, negative sense RNA virus measuring 80-120 nm in diameter, characterized by a tri-segmented genome consisting of the L segment encoding RNA-dependent RNA polymerase, M segment encoding glycoproteins Gn and Gc, and S segment encoding nucleoprotein (NP) and non-structural protein (NSs) (Yu et al. 2011). The virus is transmitted through bites from infected longhorned tick (*Haemaphysalis longicornis*) (Luo et al. 2015). The virus also has been detected in *Amblyomma testudinarium* and *Ixodes nipponensis*, revealing other potential species for SFTSV transmission (Yun et al. 2014). Additionally, cat

to human and human-to-human transmission have been documented through direct contact with blood or bodily fluids of infected patients (Kida et al. 2019; Wu et al. 2022).

The clinical manifestation of SFTS typically begins with an incubation period of 5-14 days following tick exposure, presenting initially with sudden onset of high fever, severe fatigue, and gastrointestinal symptoms including nausea, vomiting, diarrhea, and abdominal pain (Liu et al. 2014b). Laboratory findings characteristically reveal thrombocytopenia, leukopenia, and elevated liver enzymes, indicating multi-organs association (Park J. 2015). Multiple organ dysfunction syndrome (MODS) involving the liver, kidneys, heart, and lungs represents the most critical complication, often leading to fatal outcomes (Gai et al. 2012b). The case fatality rate varies depending on several factors, with elderly patients, those with underlying comorbidities, and individuals presenting with high viral loads of infection facing significantly higher mortality risks (Wang et al. 2017; Jo et al. 2022).

In domestic and wild animals, SFTSV infection has been documented across multiple species with varying clinical manifestations. Domestic cats and dogs are susceptible to SFTSV infection, with infected cats exhibiting fever, lethargy, thrombocytopenia, and occasionally fatal outcomes and capable of transmitting the virus to humans through direct contact, while seropositive dogs often remain asymptomatic or display mild symptoms and considered as a reservoir than amplifying hosts due to insufficient viremia levels (Park et al. 2019a; Saga et al. 2023; Wang et al. 2021). Livestock including cattle, goats, and sheep demonstrate high seroprevalence in endemic areas but typically present subclinical infections, though they play important roles as reservoir hosts (Huang et al. 2019; Li et al. 2014). Wild animals such as wild boar, deer, and small mammals including hedgehogs and weasels serve as natural reservoir hosts, maintaining the virus in sylvatic cycles with minimal or no clinical signs

despite sustaining viremia sufficient for tick transmission (Zhao et al. 2022; Kaneko et al. 2023). Current SFTSV treatments are restricted to supportive care due to the lack of approved antiviral therapeutics or vaccines available. However, favipiravir has emerged as a promising antiviral candidate, showing significant efficacy in clinical trials when administered early in the course of infection (Li et al. 2021; Saijo et al. 2025). This underscores the critical need for rapid diagnostic tools that enable early detection, clinical management, and implementation of infection control measures to reduce disease transmission and improve patient outcomes.

2.2. Pathogenesis and Cell Tropism

SFTSV demonstrates broad cellular tropism, infecting multiple cell types including monocytes, macrophages, dendritic cells, endothelial cells, and hepatocytes, which contribute to the systemic disease and multi-organ involvement observed in severe cases (Peng et al. 2016; Matsuno et al. 2017). Viral entry is mediated through interactions between viral glycoproteins and host cell receptors, including DC-SIGN and phosphatidylserine receptors (Tani et al. 2016; Hofmann et al. 2013), with recent studies identifying the AXL/GAS6 pathway as a critical entry mechanism involving PI3K-PLC-dependent macropinocytosis (Jin et al. 2025). However, SFTSV exhibits limited cytopathic effects in most standard cell culture systems (Yu et al. 2011). This characteristic has hindered rapid diagnostic development and fundamental virological research. Understanding SFTSV cellular tropism is crucial for elucidating disease pathogenesis and developing accessible therapies for SFTS. SFTSV ability to replicate efficiently in certain cell types while showing restricted replication in other cells suggests cell-specific factors regulating susceptibility, yet the molecular determinants of cellular permissiveness remain incompletely understood, highlighting the need for

comprehensive studies on SFTSV infectivity patterns across diverse mammalian cell lines to identify critical host factors and develop diagnostic and therapeutic strategies.

2.3. Epidemiology of SFTSV

Since its first identification in Henan Province, China in 2009 and formal virological characterization in 2011, SFTS has demonstrated rapid geographical expansion across East Asia. Mainland China bears the highest disease burden with cumulative case reports exceeding 30,000 since 2010 (Yu et al. 2011). The disease subsequently spread to neighboring countries, with Japan reporting approximately 800 cases between 2013 and 2022, primarily in Western regions with a high incident rate reported in Miyazaki prefecture (Fukushima et al. 2025). South Korea reported 2,065 cases between 2013 and 2024, and emerging reports also come from Vietnam in 2017, Pakistan in 2020, and Taiwan in 2019 (KDCA; Tran et al. 2019; Zohaib et al. 2020; Peng et al. 2020). The disease distribution strongly correlates with the habitat range of *Haemaphysalis longicornis* ticks in temperate and subtropical regions at altitudes below 1,500 meters, and peak incidence occurring during April to October coinciding with increased tick activity and human outdoor agricultural activities (Zu et al. 2022; Han et al. 2025). Spatial correlation between high livestock seroprevalence and human case clustering in agricultural communities underscores the domestic animal role in amplifying viral circulation through increased tick population densities and human exposure (Xing et al. 2016; Lu et al. 2025). Human risk factors include direct contact with infected animals, tick-infested companion animals in households, and animal husbandry activities (Liang et al. 2014; Yamanaka et al. 2020; Kaneko et al. 2023; Miyauchi et al. 2022). Human-to-human transmission particularly occurs in healthcare workers and family caregivers (Fang et al. 2021; Wu et al. 2022). The continued emergence in new geographical areas, coupled with climate

change potentially expanding tick habitats and increasing human-animal-tick contact, positions SFTSV as a pathogen requiring One Health surveillance integrating human, animal, and environmental monitoring systems (Chen et al. 2025).

The epidemiological profile of SFTS reveals complex multi-host transmission dynamics involving diverse animal populations serving as critical viral reservoirs. In China, comprehensive serological surveys conducted across multiple provinces from 2011 to 2020 demonstrated remarkably high SFTSV antibody prevalence in livestock, with seropositivity rates of 51.7-83.3% in Shandong Province, 47.2% in Henan Province, and 30-60% in Jiangsu Province, indicating widespread subclinical infections that maintain enzootic transmission cycles (Ding et al. 2014; Li et al. 2014; Huang et al. 2019). Chinese wild animal surveillance identified SFTSV RNA in rodents (3.9-8.5%), hedgehogs (12.5%), shrews (12.5%), and weasels (15-20%), with viral isolation from yellow weasel, confirming their role as competent reservoir hosts (Liu et al. 2014a; Huang et al. 2019; Gu et al. 2022; Zhao et al. 2022). In Japan, systematic serological surveys conducted in several prefectures including Oita, Ehime, Yamaguchi, and Miyazaki prefectures since 2013 revealed SFTSV antibodies in cattle, goats, wild deer, wild boar, and notably high prevalence in feral cats, with viral RNA detected in Japanese badgers (*Meles anakuma*) (Kimura et al. 2018; Matsuu et al. 2019; Hashimoto et al. 2022; Tatemoto et al. 2022; Kaneko et al. 2023). Domestic animals such as cats and dogs also show seropositivity with fatal clinical cases particularly in outdoor cats (Chen et al. 2019; Matsuu et al. 2021; Hashimoto et al. 2022). In South Korea, nationwide surveillance from 2013 identified SFTSV antibodies and RNA in cattle, goats, Korean water deer, wild boar, and small mammals (Oh et al. 2016; Rim et al. 2021; Chae et al. 2024).

2.4. Diagnosis of SFTS

Early and accurate diagnosis of SFTS remains critically challenging due to non-specific clinical presentation overlapping with other febrile illnesses such as Japanese spotted fever, scrub typhus, or other tick-borne diseases, necessitating laboratory confirmation (Watt and Parola. 2003; Liu et al. 2014b; Wu et al. 2016; Noguchi et al. 2018). The current gold standard for confirmatory diagnosis uses real-time reverse transcription polymerase chain reaction (RT-PCR) targeting viral segments, demonstrating high sensitivity and specificity during acute phase within the first week post-symptom onset (Yoshikawa et al. 2014; Yun et al. 2016a). However, conventional RT-PCR requires expensive equipment, costly reagents, and time-consuming, restricting implementation in resource-limited rural, endemic areas. Serological diagnosis using ELISA or immunofluorescence assay serves as complementary diagnosis, though seroconversion may be delayed or absent in fatal cases with overwhelming viremia (Spivak et al. 2010). Virus isolation using Vero E6 cells, while providing definitive diagnosis, requires BSL-3 facilities, reducing its practical utility routine for clinical use (Yu et al. 2011; Meechan et al. 2020). Commercial diagnostic kits in East Asia lack international standardization, while cross-reactivity with other *Phleboviruses* poses diagnostic challenges (Matsuno et al. 2013; Rolando et al. 2024).

These diagnostic limitations become critical in rural endemic areas where delayed diagnosis correlates with increased mortality, as primary healthcare facilities often lack molecular diagnostic capabilities, resulting in empirical treatment based on clinical suspicion and basic laboratory findings (Penon-Portmann et al. 2020; Kumar et al. 2025). Point-of-care testing (POCT) requirements include rapidity, simplicity, affordability, and ability to use whole sample without complex steps is needed in limited-resources setting (Shaw JLV. 2015;

Salayer et al. 2017). Loop-mediated isothermal amplification (LAMP) has emerged as a promising alternative, offering rapid amplification at constant temperatures (60–65°C) without thermal cycle equipment, with visual detection through turbidity or colorimetric changes, and sensitivity comparable to RT-PCR while being more tolerant of sample inhibitors (Notomi et al. 2000; Nagamine et al. 2002). Development of direct sample RT-LAMP assays eliminating RNA extraction represents significant advancement toward practical POCT of SFTSV diagnosis, potentially enabling early detection in remote areas, rapid case triage, and timely implementation of supportive care and infection control measures crucial for improving patient outcomes and preventing nosocomial transmission in this emerging viral disease with substantial public health impact (Sano et al. 2021; Hashimoto et al. 2024).

2.5. Research Gap

Current SFTSV diagnosis relies on laboratory equipment, including real-time RT-PCR and virus isolation which require equipped facilities. This reliance on advanced laboratory facilities creates a diagnostic accessibility gap in resource-limited, rural areas where SFTSV is endemic, particularly in mountainous areas where agricultural communities face the highest infection risk. In addition to these diagnostic barriers, SFTSV research is further hindered by fundamental knowledge gaps. Most SFTSV research has focused on narrow range of conventional cell lines, leading to incomplete characterization of viral cellular tropism. The absence of systematic evaluation across diverse mammalian cell lines created knowledge gaps regarding species-specific susceptibility patterns, optimal cell culture systems for viral studies, and virus-host interaction that determines cellular susceptibility and pathogenesis.

**3. CHAPTER 1. Reverse Transcription Loop-Mediated Isothermal Amplification Assay
Using Samples Directly: Point-of-Care Detection of Severe Fever with Thrombocytopenia
Syndrome Virus**

3.1. Abstract

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne disease caused by the SFTS virus (SFTSV). A rapid and cost-effective point-of-care testing detection system is important for the early diagnosis of SFTS. Herein, we developed a ready-to-use dried reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the direct detection of SFTSV in clinical samples. The assay enables simple, RNA-extraction-free detection using heat-treated serum or plasma, followed by a 30 min incubation at 65°C. The results are visually interpreted through the color emitted, which can be observed under LED light. The established assay demonstrated detection sensitivity for SFTSV at 10⁴ copies/μL and was effective in identifying infections in cats. Despite being less sensitive than real-time RT-PCR, this dried RT-LAMP method offers a rapid, cost-effective alternative suitable for point-of-care use, particularly in remote or resource-limited settings. The simplified workflow and visual readout make it a practical tool for the early detection and daily surveillance of SFTSV in animals.

3.2. Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is a new emerging tick-borne infectious disease. SFTS was first identified in China in 2009 (Yu et al. 2011) and has since been reported in Japan, South Korea, Vietnam, Myanmar, Thailand, and Pakistan (Kim et al. 2013; Takahashi et al. 2014; Tran et al. 2019; Zhao et al. 2020; Ongkittikul et al. 2020; Zohaib et al. 2020). The causative agent of SFTS is the SFTS virus (SFTSV) (Yu et al. 2011), which is currently classified as a *Bunyavirales*, family *Phenuiviridae*, genus *Bandavirus*, and species *Bandavirus dabiense*. SFTSV is a single-stranded, negative-sense RNA virus whose genome comprises three RNA segments: large(L), medium (M), and small (S). These encode an RNA-dependent RNA polymerase (RdRp), glycoproteins (Gn and Gc), and the nucleoprotein (N)

and non-structural proteins (NSs), respectively. The primary clinical signs of SFTS are fever, thrombocytopenia, gastrointestinal disorders, and leukocytopenia, which can lead to multi-organ failure, with a fatality rate of 10–16% (Yu et al. 2011; Zhang et al. 2012). Currently, there are no specific treatment drugs or licensed vaccines available for SFTS, making it a significant medical problem. However, the detailed pathogenesis of this disease remains unknown (Takahashi et al. 2014). SFTSV is transmitted primarily via ticks such as *Haemaphysalis longicornis* (Kim et al. 2013). SFTSV infections have been identified in wild animals (wild boars and wild deer), livestock (cattle, pigs, and goats), and companion animals (cats, dogs, and captive cheetahs) (Tabara et al. 2016; Hwang et al. 2017; Yu et al. 2018; Kimura et al. 2018; Chen et al. 2019; Kida et al. 2019). In particular, many feline cases of SFTS have been reported in Japan (Matsuo et al. 2019; Ando et al. 2021; Irie et al. 2022), and transmission of SFTSV from animals to humans is considered to be an important public health concern (Liang et al. 2014; Yamanaka et al. 2020; Miyauchi et al. 2022).

The detection of SFTSV genes is a standard diagnostic method for SFTS in humans and animals (Yu et al. 2011; Ando et al. 2021). Real-time reverse transcription polymerase chain reaction (RT-PCR) has been applied to detect SFTSV RNA in blood, serum, and oral swab samples taken from humans and animals (Sun et al. 2012; Ando et al. 2021). RT-PCR requires advanced and high-throughput equipment and is primarily performed at specialized institutions such as hospital laboratories or institutes, and for small animal hospitals, it takes time to transport samples and receive diagnostic results. Therefore, a point-of-care testing (POCT) method other than RT-PCR is required for the rapid diagnosis of SFTSV infection.

Loop-mediated isothermal amplification (LAMP) is a DNA amplification technique that enables rapid and accurate gene detection. LAMP can be performed at a constant

temperature for less than an hour, with high sensitivity and specificity. In addition, LAMP can be performed even in unprocessed samples, including whole blood, serum, urine, and stool samples, which may inhibit DNA amplification with PCR (Francois et al. 2011). Owing to its simplicity and minimal equipment, the LAMP method can be used as an effective and cost-efficient molecular diagnostic method in areas with limited resources (Notomi et al. 2000).

Previous studies have reported that the RT-LAMP system for SFTSV provides useful insights into the possibility of POCT for SFTS (Yang et al. 2012; Xu et al. 2013; Baek et al. 2018; Lee et al. 2020b; Sano et al. 2021; Jang et al. 2022; Ishijima et al. 2023). In these studies, the RT-LAMP reaction was evaluated using SFTSV RNA extracted from samples. Ishijima et al. (2023) directly demonstrated SFTSV RT-LAMP using clinical samples mixed with an RNA extraction reagent (Ishijima et al. 2023; Sano et al. 2021). However, result determination is still dependent on an adequate machine, such as a real-time fluorometer.

Herein, we developed a diagnostic technique using a dried RT-LAMP system that can be performed with a single-tube, sample-in-answer-out format without requiring sophisticated equipment, with the final goal of creating a simpler method for detecting SFTS based on previous studies. Our approach allows the direct detection of serum or plasma sample of SFTS without an RNA extraction step, for which the outcomes can be observed by a simple observation under an LED light. This dried-RT-LAMP system can be used to detect SFTSV in limited-resource clinics without requiring advanced equipment.

3.3. Material and method

3.3.1. Viruses and cells

The SFTSV YG-F33 and YG-F185 strains were propagated in Vero E6 cells in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies Corporation,

Grand Island, NY, USA) supplemented with 2% fetal bovine serum (FBS) (Biosera, Cholet, France; BioNordika AS, Oslo, Norway). Vero E6 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). The supernatant of SFTSV-infected cells was stored at -80°C as a viral stock. All experiments using infectious SFTSV were performed in a biosafety level 3 laboratory at Yamaguchi University.

3.3.2. Samples

Clinical serum and plasma samples from cats were provided by veterinary clinics in Japan and sent to our laboratory for the diagnosis of SFTS. Plasma samples of experimental mice were collected from SFTSV-infected IFN α / β R $^{-/-}$ (A129) mice at 0 to 4 days post-infection (dpi).

3.3.3. Viral RNA extraction

SFTSV viral RNA was extracted from the viral stock using ISOGEN-LS (NIPPONGENE Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. A total of 60 μL of RNA was extracted from 140 μL of serum or plasma from cat patients and SFTSV-infected mice using a QIAamp[®] Viral RNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions.

3.3.4. Real-Time RT-PCR

Real-time RT-PCR was performed to measure the SFTSV copy number. A two-primer set, comprising 965F (5'-GCRAGGAGCAACAARCAAACATC-3') and 1069R (5'-GCCTGAGTCGGTCTTGATGT C-3'), and probe FAM/5'-CTCCCRCCC-3'/ZEN/5'-TGGCTACCAAAGC-3' (Integrated DNA Technologies) were used to amplify and detect the L segment of SFTSV. Real-time RT-PCR was performed using the CFX Connect[™] Real-Time System (BIO-RAD) software (v. 2.3). Real-time reactions were carried out

using a TaKaRa One Step PrimeScript™ RT-PCR (Perfect Real Time) Kit (Takara Bio Inc., Shiga, Japan), as follows: reverse transcription at 42 °C for 5 min, initial denaturation at 95 °C for 10 s, 40 cycles of denaturation at 95 °C for 5 s, and an annealing and extension step at 60 °C for 34 s. The viral copy numbers were determined as the ratio of the copy numbers to the standard control prepared from a SFTSV gene-cloned plasmid vector, as previously studied (Hayasaka et al. 2015).

3.3.5. Preparation of the Dried RT-LAMP Assays in a PCR Tube

Dry-format LAMP reagents were prepared using an air-drying technique, as reported previously (Hayashida et al. 2019). For the LAMP primer sets used to detect SFTSV, previously reported primer sets targeting the L segment were used (Baek et al. 2018). Briefly, 3.85 µL of enzyme mix comprising 1.6 µL of 2 M trehalose (Fujifilm Wako Pure Chemical, Osaka, Japan), 1.4 µL of 25 mM dNTPs (Nippon Gene, Tokyo, Japan), 0.25 µL of WarmStart RTx reverse transcriptase (New England Biolabs Inc., Ipswich, MA, USA), 0.05 µL of Bst 2.0 WS DNA polymerase (120 U/µL) (New England Biolabs Inc., Ipswich, MA, USA), 0.25 µL of Bst 2.0 WS DNA polymerase (8 U/µL), and 0.3 µL of RNase inhibitor (Takara Bio Inc., Shiga, Japan) were applied inside the flat tube lid of the 0.2 mL PCR tube (Watson, Kobe, Japan). Two different concentrations of Bst 2.0 WarmStart DNA polymerase were used to adjust the glycerol amount to maintain the effective drying time and stability of the enzyme in the assay. A total of 3 µL of LAMP primers mixed with colorimetric indicator developed by Hayashida et al. 2019, comprising 0.4 µL of 100 mM FIP and BIP (forward and backward inner primers), 0.2 µL of 100 mM LF and LB (loop primers), 0.05 µL of 100 mM F3 and B3 (outer primers), 0.7 µL of 2 M trehalose, and 1 µL of colorimetric indicator (CFI; 3 mM hydroxyl-

naphthol blue (DOJINDO LABORATORIES, Kumamoto, Japan) and 0.35% v/v GelGreen (10,000X stock solution in DMSO, Biotium, CA, USA), were placed into the bottom of the tube. To dry the liquid reagents, the tubes were placed in a glove box filled with dried air produced by an ultralow dew point air dryer (QD20-50; IAC Co., Kawasaki, Japan) for 12 h. The in-house-dried LAMP reagent for the lids and tubes was stored separately in an aluminum bag with silica gel to avoid light exposure at room temperature until use.

3.3.6. RT-LAMP reaction

For the LAMP reaction, 23 μ L of LAMP reaction buffer, comprising 20 mM Tris-HCl (pH 10.0), 50 mM KCl, 6 mM MgSO₄, 10 mM (NH₄)₂SO₄, and 0.1% Triton X-100, was applied into the tube with 2 μ L of the reaction template RNA or lysate. Triton X-100 was added to lyse the viral membrane and enhance the detection sensitivity. The tube was then placed upside down for 2 min until the enzyme mix on the tube lid was reconstituted and then spun down. The LAMP reaction was performed using a real-time PCR machine (RotorGene6000; Corbett Life Science, Sydney, Australia) to monitor the reaction and experimental setup, allowing us to determine the reaction time threshold. For the experiment using viruses and clinical samples, a dried RT-LAMP reaction was performed by incubating the RT-LAMP tube at 65 °C for 30 min using a BI-516S Block Incubator (Astec Co., Ltd., Fukuoka, Japan). The LAMP products were detected directly by the naked eye and under LED light using a handmade, battery-operated LED illuminator (Hayashida et al. 2015). DNA amplification was monitored by observing the fluorescence from the dsDNA-specific dye CFI contained in the dried RT-LAMP mixes. Staining enabled the detection of amplified DNA under an LED light with a wavelength of 500 nm. A portable, battery-operated handheld LED illuminator was built to facilitate result

evaluation (Figure 3.1); hence, there was no need to use an additional advanced device to read the results from the RT-LAMP reaction. Under the naked eye, the positive tubes showed a light blue color, and the negative tubes showed a light purple color. A greenish-yellow color emitted from the RT-LAMP tube under LED light indicated a positive result, whereas an orange color indicated a negative result (Figure 3.1).

3.3.7. Heat inactivation

Measures of 2 μL of the SFTSV stock samples (10^7 focus forming unit (ffu) per mL) were boiled with LAMP reaction buffer in 25 μL total reaction volume including 2×10^4 ffu of SFTSV at 99 °C for 10, 20, and 30 min. Non-heat-treated samples were used as the controls. All tests were performed duplicate. Subsequently, 2 μL of the serum or plasma samples obtained from the cats or mice were mixed with 23 μL of LAMP reaction buffer in a 1.5 mL micro-centrifuge tube. The sample mix was treated with heat inactivation at 99 °C for 10 min. After heat inactivation, the tube was spun down briefly, and a total of 25 μL of reaction volume was transferred into the dried RT-LAMP tube. The dried RT-LAMP tubes were inverted several times until the dried reagent was reconstituted into the sample mix. The tubes were then placed upside down and incubated for 2 min at room temperature. The LAMP reaction was performed with a dry bath at 65 °C for 30 min. The SFTSV copy numbers in the serum and plasma samples were measured beforehand using a real-time RT-PCR assay, and the correlations between the Ct values, copy numbers, and RT-LAMP assay results were compared. Purified SFTSV RNA was used as a positive control, and RNase-free water was used as a non-template control.

3.4. Results

3.4.1. Sensitivity and detection limit analysis of the SFTSV-Dried RT-LAMP assay

Purified viral RNA prepared from the SFTSV samples was used to monitor amplification by light blue color change and fluorescence. RNA was successfully amplified, and a positive reaction was observed within 30 min. Subsequently, using a 10-fold serial dilution of purified SFTSV RNA, the detection limit of the dried RT-LAMP assay was evaluated. The detection limit of the dried RT-LAMP assay was 10^4 copies/ μ L, because no positive reaction was observed in 10^3 copies/ μ L (Figure 3.2).

3.4.2. Heat inactivation test

To establish a safe and rapid diagnostic test workflow, we tested the SFTSV stock samples inactivated with simple boiling for 10 min in the LAMP reaction buffer that included Triton X-100. In general, it seems that boiling for 10 min inactivates infectious viruses, and our unpublished data indicate that 99 °C for 10 min significantly inactivates infectious SFTSV. Positive reactions of heated SFTSV samples were clearly detected by color emissions under LED light, whereas those light blue color changes were not distinctly observed (Figure 3.3), suggesting that direct naked eye observation is not suitable for SFTSV samples without RNA extraction. Interestingly, the heat-treated samples performed better than the unheated samples (Figure 3.3). Therefore, the simple boiling method is likely to be useful not only to minimize the risk of infection by heat inactivating but also to eliminate the RNA extraction step without additional reagents. For further studies, we conducted the LAMP assay for clinical samples using heat inactivation at 99 °C for 10 min and observed the positive reactions under an LED light.

3.4.3. Application of the Dried RT-LAMP assay to clinical samples

To test the clinical samples, we first confirmed the RT-LAMP assay using plasma samples from SFTSV-infected mice. Copy numbers of the mouse samples were determined using real-time RT-PCR. Positive reactions were observed compared to the non-template control after the completion of the reaction.

Positive reactions with color emission under LED light were clearly detected in the sample numbers (Day 2—118; Day 2—123; Day 3—131; Day 3—134; Day 4—124; Day 4—133), corresponding to high copy numbers with more than 10^4 copies; however, those emissions were comparatively weak in other sample numbers: Day 2—124; Day 3—132 (Figure 3.4). Positive reactions of color emission under LED light were not observed in the other samples with fewer than 10^2 copies (Figure 3.4). From these results, it was suggested that, for the SFTSV-infected mouse samples, the dried RT-LAMP-positive results observed by color emission mostly appeared on days 2–4 post-infection, showing a viral copy number above 10^4 copies/reaction.

Next, we examined the positive reactions of SFTSV-suspected cat samples with high copy number (more than 10^4 copies) confirmed by real-time RT-PCR. The dried RT-LAMP assay clearly detected SFTSV positive-confirmed samples with color emission via LED light (Figure 3.5). All the SFTS-negative samples did not show positive reactions of color emission under LED light (Figure 3.5). These observations suggest that the dried RT-LAMP assay under LED light is useful for SFTS diagnosis using cat samples as a POCT.

3.5. Discussions

In this study, we developed a dried RT-LAMP assay for SFTSV using serum and plasma samples for point-of-care testing without RNA extraction. The assay contains a liquid LAMP

reaction buffer and dried reagents in the reaction tubes, which can be maintained at room temperature or 4 °C for extended periods of time. We further used an emission colorimetric indicator to simplify the interpretation of the results. Negative and positive results of cat samples can be easily distinguished under LED light with a simple, handmade, battery-operated handheld LED illuminator.

Interestingly, we found that the heat inactivation test at 99 °C for 10 min increased the sensitivity of the assay for SFTSV samples without RNA extraction. The LAMP reaction buffer contained Triton X-100, and previous research has suggested that a detergent containing 0.1% Triton X-100 in the buffer can accelerate viral membrane lysis during boiling (Hayashida et al. 2017; Kapalamula et al. 2021). Several studies have shown that boiling samples with endogenous RNase-reducing or lysis agents to inactivate endogenous RNase can improve the sensitivity and specificity of an assay (Anahtar et al. 2021; Hayashida et al. 2023). In our unpublished study, which investigated the inactivation effects of various reagents with high temperature, we showed that a reagent including 0.1% Triton X-100 efficiently inactivated the infectious SFTSV in clinical samples from SFTSV-infected animals at 99 °C for 10 min. Therefore, it was considered that Triton X-100 in the LAMP reaction buffer would lyse the viral membrane and expose the viral genome to the LAMP reagent. Preheating the sample and LAMP reaction buffer containing Triton X-100 at a boiling temperature for 10 min provides a simple yet effective method for replacing RNA extraction that can be performed anywhere.

Compared to the previous LAMP SFTS, which detects purified RNA from a sample, the current approach eliminates the RNA extraction step, simplifying the method. Alternative, non-commercial approaches to RNA isolation, such as those involving magnetic beads or a silica gel matrix, require specialist knowledge and are difficult to adopt for on-site screening

(Thi et al. 2020). Another method using the Loopamp viral RNA extraction solution with preheat treatment at 90 °C for 1 min has been used to simplify the RNA extraction step (Hayasaka et al. 2015; Ishijima et al. 2023). Although the procedure for RNA extraction using this reagent is simple and rapid, it is expensive.

Using a colorimetric RT-LAMP mixture, the LAMP results can be visually determined by the naked eye via a color change (Baek et al. 2018). Based on a combination of one-pot colorimetric visualization and a pocket warmer reaction platform, the RT-LAMP system can detect approximately 10 viral genome copies of SFTSV (Baek et al. 2018). The detection of SFTSV using one-step RT-LAMP with the fluorescent detection reagent SYBR Green I can be visualized under UV light (Xu et al. 2013). However, these methods still require RNA extraction. Our dried RT-LAMP assay also clearly showed positive reactions by naked eye observation with light blue color changes for extracted viral RNA. However, the naked eye observation was not suitable for heated SFTSV samples without RNA extraction. Instead, those positive observations were clear under the LED illuminator that was handmade at a low cost, suggesting that it could be useful for clinical sites. It is of note that the viral copy number of SFTSV that can be detected using the dried RT-LAMP assay is 10^4 copies/ μ L of sample or 2×10^4 copies per reaction. The sensitivity of our dried RT-LAMP assay using clinical samples was similar to that of the purified SFTS RNA.

In mice samples, several samples that tested positive using real-time RT-PCR exhibited negative interpretations despite the viral copy being above the detection limit, 10^4 copy/reaction. Meanwhile, in the cat samples, after confirmation by real-time RT-PCR, all the positive samples showed positive results, and all the negative samples showed negative results according to the RT-LAMP assay. These results show consistency within our dried RT-LAMP

assay. In clinical samples, direct visual interpretation was compromised, as the colorimetric change was not clearly observed through naked eye despite positive results being distinctly evident under LED illumination. This result could be due to the presence of blood cell pellets within the samples, which interfered with visual color assessment using direct naked eye observation. Previous studies have demonstrated that, while blood components can impair naked eye color interpretation, they do not affect fluorescence detection (Hayashida et al. 2015). For field applications, we suggest that the result interpretation should be performed by both the direct naked eye and fluorescence observations, given the variable quality of the clinical specimens that cannot be standardized in remote settings. Therefore, the handmade, battery-operated, handheld LED illuminator was made previously (Hayashida et al. 2015) to facilitate the detection in the field, enhancing the capability of field detection. This approach maintains diagnostic accuracy while minimizing the need for sophisticated laboratory equipment, making the assay suitable for resource-limited environments where advanced instrumentation may not be available.

SFTS-suspected samples should be sent to a recommended institution to confirm the diagnosis. Although the sensitivity and specificity of SFTSV RT-LAMP may not be high, the RT-LAMP assay could be very useful for rapid diagnosis in hospitals for primary diagnosis before confirmation with laboratory diagnosis in an institution. Furthermore, a positive result reflects a high viral load in the sample, indicating the risk of transmission between humans and animals.

RT-LAMP has been used to detect many RNA viruses, including Dengue, Japanese encephalitis, SARS-CoV-2, MERS-CoV, tick-borne encephalitis virus, and influenza A (Parida et al. 2005; Toriniwa and Komiya. 2006; Jayawardena et al. 2007; Hayasaka et al. 2013; Lee

et al. 2017; Raddatz et al. 2023). The requirement of a facility to diagnose SFTS makes it difficult to acquire disease surveillance data, and the rapidity of diagnosis also has an impact on the medical care treatment for the patient (Zhang et al. 2022a; Park et al. 2019b). Many clinics rely on large institutions with adequate facilities, since diagnosing SFTS in patients requires specialized diagnostic equipment. As such, a quick and simple one-pot diagnostic method that can be performed in clinics with minimal equipment is crucial for the early detection and diagnosis of SFTSV. Owing to its quick turnaround time, RT-LAMP is a promising alternative to RT-PCR for detecting SFTSV in clinical samples.

3.6. Conclusions

This study demonstrated that our dried RT-LAMP assay can serve as an alternative diagnostic tool for SFTSV detection using serum and plasma samples, providing a point-of-care testing (POCT) method. The dried RT-LAMP for the SFTSV assay requires only a heat block, a portable handmade LED illuminator, and a LAMP tube that has already been mixed with the dried reagents (substrate, primer, and enzyme) to carry out simultaneous RNA-and DNA-specific amplifications. We hope that this study will facilitate the implementation of an affordable diagnostic method using the RT-LAMP assay in settings where the use of commercial kits is not possible, such as in clinics with limited access to advanced facilities. As such, this approach could help address several issues associated with the accessibility of reliable, affordable, quick, and efficient diagnostic instruments for detecting SFTSV in animals, though we suggest that samples should be sent to recommended institutions to confirm diagnoses. Nonetheless, this assay will have significant utility in areas with limited resources, particularly when rapid diagnostic outcomes are required.

3.7. Figures of Chapter 3.



Figure 3.1. Handmade-battery operated handheld LED illuminator. The handmade LED illuminator emits 500 nm wavelength light to detect the LAMP fluorescence reaction. The portable illuminator is easy to assemble and suitable for clinical site.

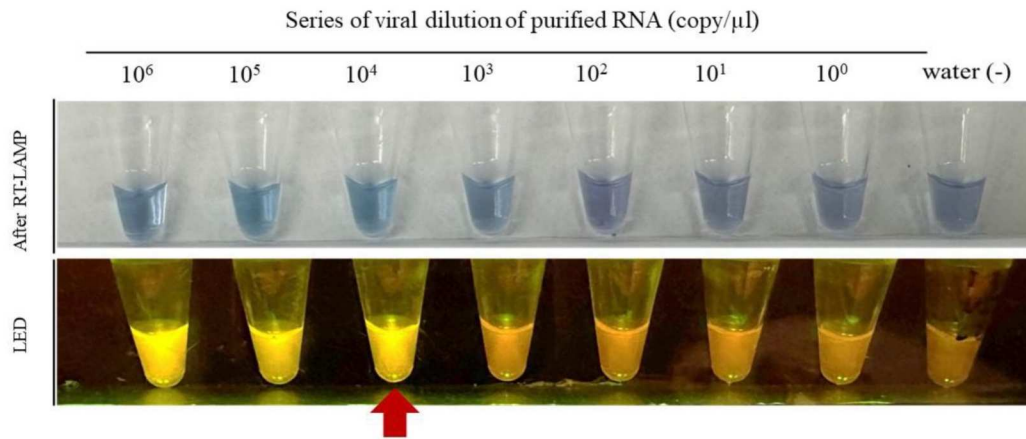


Figure 3.2. Analytical sensitivity of the dried SFSTV RT-LAMP assay. SFSTV viral stock was extracted for RNA, and viral copy number was measured using real-time RT-PCR. Ten-fold dilutions of SFTSV RNA were amplified via RT-LAMP and detected using a colorimetric indicator. The assay detected the SFTSV RNA up to 10^4 copies/ μ L (red arrow). The assay results could be observed with light blue color changes (**upper panel**) and greenish-yellow color emissions under an LED light (**bottom panel**).

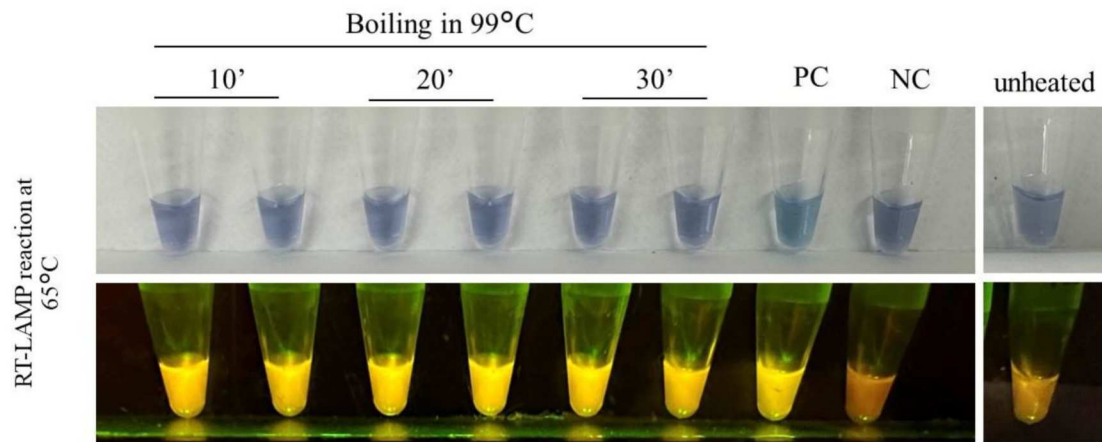


Figure 3.3. Heat inactivation test of the RT-LAMP assay. The RT-LAMP results could be observed with the naked eye (**upper panel**) and under an LED light (**bottom panel**). After being treated with boiling at 99 °C for 10, 20, and 30 min, the SFTSV sample including 2×10^4 ffu of SFTSV was tested with dried RT-LAMP assay. The results observed under LED light showed that heat treatment of samples increased the sensitivity of the dried RT-LAMP assay compared to the unheated samples. SFTSV viral RNA and distilled water were used as positive control (PC) and negative control (NC), respectively.

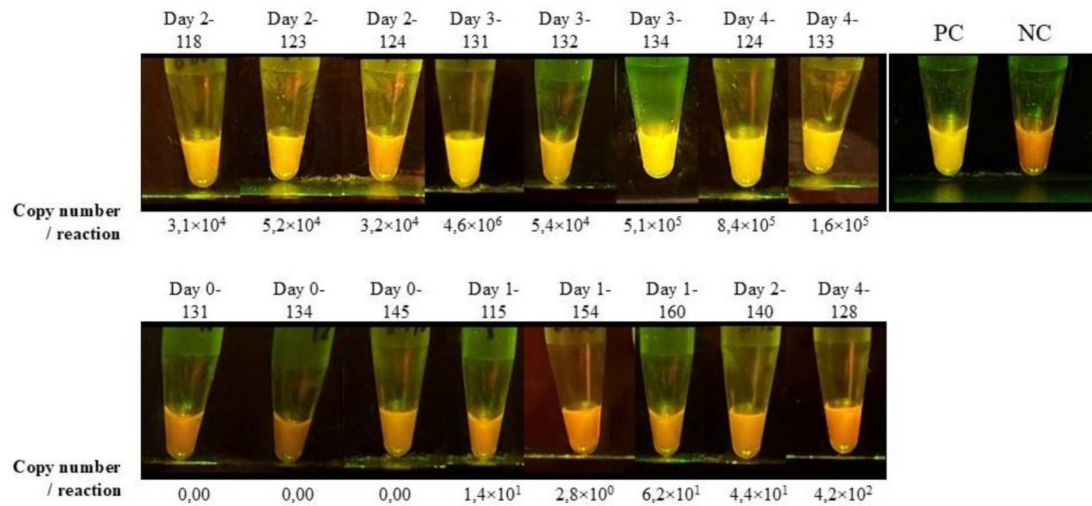


Figure 3.4. Evaluation of the SFTSV RT-LAMP assay in the SFTSV experimental mice plasma samples. Positive results (**upper**) indicated by greenish-yellow color under LED light and negative results(**bottom**) indicated as orange under LED light. The same samples were also measured for the viral copy number using real-time RT-PCR, and the numbers are shown in the figure. In the mouse samples, positive results were achieved in samples with high viral copy numbers. Samples with a copy number less than 2×10^4 copies/reaction provided negative results. SFTSV viral RNA and distilled water were used as positive control (PC) and negative control (NC), respectively.

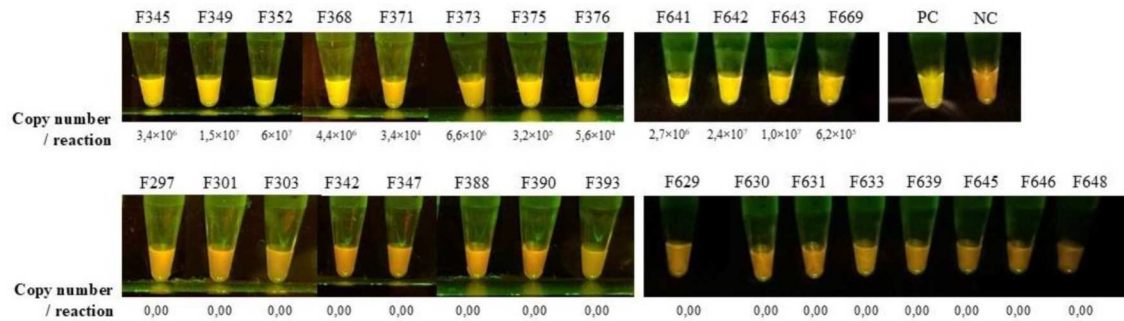


Figure 3.5. Evaluation of the SFTSV RT-LAMP assay in the SFTS-suspected cat serum and plasma. Positive results indicated by a greenish-yellow color emission (**upper panel**) and negative results without emission (**bottom panel**) under LED light. The same samples were also measured for the viral copy number using real-time RT-PCR, and the numbers are shown in the figure. SFTSV viral RNA and distilled water were used as positive control (PC) and negative control (NC), respectively.

**4. CHAPTER 2. Different Susceptibility of Mammalian Cell Lines to Severe Fever with
Thrombocytopenia Syndrome Virus Infection**

4.1. Abstract

Severe Fever with Thrombocytopenia Syndrome (SFTS) is an emerging tick-borne infectious disease that poses a significant public health threat. SFTS virus (SFTSV) has a broad host range, including humans, cats, and natural reservoir species. Therefore, cultured cell lines derived from different mammalian species are useful for understanding the susceptibility of SFTSV in hosts. In this study, we evaluated pathogenicity and infectivity, focusing on cytopathic effect (CPE) induction and growth kinetics of SFTSV in several mammalian cell lines, including our original tiger-derived TLT, wild deer-derived DFKT and DFLT, and hedgehog-derived HHoVT. Following SFTSV infection, TLT, CRFK (cat), FCWF-4 (cat), and CPK (porcine) cells exhibited CPE, whereas Vero E6 (monkey), A549 (human), BHK-21 (hamster), DFKT, DFLT, and HHoVT cells did not. Infectious viral yields in the supernatants of TLT, CRFK, FCWF-4, Vero E6, and BHK-21 were higher than those of CPK, A549, DFLT, and DFKT. SFTSV infection in hedgehog-derived HHoVT cells was very limited. These observations suggest that features such as viral CPE and virus yield following SFTSV infection depend on cell type. It is noteworthy that TLT formed clear plaques that were easy to count, indicating that TLT cells are useful for the titration of infectious SFTSV by plaque-forming assay. Our results provide useful information and tools for further elucidating the mechanisms of SFTSV infectivity, proliferation, and pathogenicity using in vitro models.

4.2. Introduction

Severe Fever with Thrombocytopenia Syndrome (SFTS) is an emerging tick-borne disease caused by the SFTS virus (SFTSV), which is currently classified within the order *Bunyavirales*, family *Phenuiviridae*, genus *Bandavirus*, and species *Bandavirus dabieense* (ICTV n.d.). SFTSV was first reported in China and has been identified in Japan, South Korea, Taiwan, Vietnam, Thailand, Myanmar and Pakistan (Yu et al. 2011; Kim et al. 2013; Takahashi

et al. 2014; Liu et al. 2014b; Tran et al. 2019; Ongkittikul et al. 2020; Win et al. 2020; Zohaib et al. 2020). SFTSV is mainly transmitted to humans by ticks such as *Haemaphysalis longicornis* and *Amblyomma testudinarium* (Yu et al. 2011; Yun et al. 2014; Zhao et al. 2020). Human-to-human transmission has also been reported through direct exposure to infected blood or bodily fluids (Gai et al. 2012a; Fang et al. 2021).

Clinical manifestations of SFTS include high fever, decreased platelet and white blood cell counts (thrombocytopenia and leukopenia), and gastrointestinal symptoms such as vomiting, nausea, and diarrhea (Seo et al. 2021). In severe cases, the disease may lead to multi-organ failure with mortality rates of up to 30% (Gai et al. 2012b; Park J. 2015). Despite its significant public health impact, the pathogenesis of SFTS remains incompletely understood, and neither vaccines nor specific antiviral therapies are currently available.

SFTSV has been maintained in nature through amplifying hosts and small mammals such as rodents and shrews as reservoirs (Liu et al. 2014a; Zhao et al. 2022). SFTSV infections have been identified in various animals such as wild boars, wild deer, cattle, pigs, goats, cheetahs, dogs, and cats (Tabara et al. 2016; Hwang et al. 2017; Yu et al. 2018; Kimura et al. 2018; Chen et al. 2019; Kida et al. 2019; Matsuu et al. 2019; Ando et al. 2021; Irie et al. 2022). In particular, many cat cases of SFTS have been reported in Japan, and SFTSV infections from animals to humans have been reported, suggesting that SFTS is an important public health concern as an emerging zoonotic disease (Liang et al. 2014; Yamanaka et al. 2020; Miyauchi et al. 2022).

In vitro models using cultured cells have been used as a crucial tool to elucidate the infectivity, pathogenicity, viral tropism and host–pathogen interactions of pathogenic viruses. SFTSV demonstrates variable infectivity across various cell types derived from human and

animals (Zheng et al. 2025). Human cell lines derived from various organs such as lungs, liver, and kidney are susceptible to SFTSV infection (Hofmann et al. 2013). Diverse animal cell lines such as Vero, COS7 (Tani et al. 2016), DH82 (canine macrophage-like) and L929 (murine fibroblasts) cells are also permissive to SFTSV infection (Yu et al. 2011). Infectious susceptibility of SFTSV depends on cell type; for example, lymphocyte cell lines showed lower susceptibility to SFTSV than adherent cell lines (Tani et al. 2016). Also, cytopathic effects (CPE) due to SFTSV infection appear to depend on cell type (Sun et al. 2015; Matsuno et al. 2017).

An infectious cell culture system is also essential to quantify the SFTSV titer. Viral titrations have been achieved through various methodologies such as plaque-forming assay (PFA), focus-forming assay (FFA), tissue culture infectious dose (TCID₅₀), immunofluorescence assay (IFA), and quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Takahashi et al., 2014; Yoshikawa et al. 2014; Hayasaka et al. 2015; Taniguchi et al. 2017). In general, FFA is preferred for titrating infectious SFTSV, because SFTSV infection often resulted in limited CPE in most cell lines (Hayasaka et al. 2016). FFA provides enhanced sensitivity through antibody-based detection in infected cells; however, the results depend on the quality of the antibody, and the methods are generally time-consuming and costly compared with PFA (Cooper. 1961; Stone et al. 2022).

The differential susceptibility patterns observed among various mammalian cells suggest that cellular factors play crucial roles in determining infection outcomes such as viral replication efficiency and CPE appearance (Matsuno et al. 2017; Cao et al. 2021; Dighe et al. 2022). Therefore, the selection of appropriate cell lines and the establishment of in vitro

models are fundamental procedures to elucidate the properties of SFTSV infectivity and pathogenicity.

This study aims to systematically evaluate and compare susceptibility to SFTSV infection in multiple mammalian cell lines, including our original cell lines derived from tiger, wild deer, and hedgehog. Our results will provide updated information on useful cell cultures for in vitro SFTSV studies to enhance our understanding of SFTSV cellular tropism.

4.3. Material and method

4.3.1. Cells and virus

Vero E6 (African green monkey kidney) and BHK-21 (baby hamster kidney-21) cells were kindly provided by Dr. Hiroaki Kariwa, Hokkaido University, and have been maintained in our laboratory. FCWF-4 (feline fetal macrophage), CRFK (Crandell-Rees feline kidney), A549 (human alveolar basal epithelium), and CPK (porcine kidney) cell lines were obtained from the American Type Culture Collection (ATCC). Mammalian cell lines used in this study are listed in Table 4.1. TLT, DFKT, DFLT, and HHoVT cells were originally established in our laboratory from tiger liver, wild deer kidney, wild deer liver, and hedgehog ovary, respectively (Shimoda et al. unpublished). Briefly, the cells were immortalized by introducing Large T antigen of Simian virus 40 and have cultivated more than 40 passages. All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies Corporation, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Co., St. Louis, USA). The SFTSV Tk-F123 and Ng-F264 strains isolated from SFTS cases (Wulandari et al. 2025) were propagated in Vero E6 cells, and the stock viruses were stored at -80°C until use. All experiments involving infectious SFTSV were conducted in a Biosafety Level-3 facility at

Yamaguchi University, Japan, in accordance with institutional biosafety guidelines and national regulations.

4.3.2. FFA of SFTSV

FFA was conducted according to the methods referred to a previous study (Hayasaka et al. 2015). Briefly, Vero E6 cells were seeded into 96-well plates and inoculated with two-fold serial dilutions of SFTSV. Following a 90 min incubation at 37 °C, the SFTSV-infected cells overlaid with 1% methylcellulose (MC) DMEM with 2% FBS and incubated at 37 °C with 5% CO₂ for three days. After fixation with 4% paraformaldehyde, viral foci were detected using anti-SFTSV nucleoprotein monoclonal antibodies (Zhang et al. 2022b), followed by treatment with a goat anti-mouse IgG conjugated with horseradish peroxidase (American Qualex International, Inc., California, USA). Visualization of viral foci was performed by adding a substrate mixture of 3,3'-diaminobenzidine (Fujifilm Wako Pure Chemical Corp., Osaka, Japan) in distilled water along with 0.1% hydrogen peroxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Viral titers were measured as focus-forming units per milliliter (FFU/mL).

4.3.3. SFTSV infections in cell lines

Each cell line was seeded in 24-well plates at a density of 5×10^4 to 1×10^5 cells/well and incubated at 37 °C with 5% CO₂ until they reached confluence. The confluent cells were inoculated with SFTSV Tk-F123 at a multiplicity of infection (MOI) of 0.01. The formation of CPE was observed daily. The supernatants were collected daily from 0 to 7 days post-infection (dpi). Collected supernatants were subjected to viral titration using FFA.

4.3.4. PFA of SFTSV

PFA was performed using CRFK, FCWF-4, and TLT cells. Cells were seeded in 24-well plates in DMEM supplemented with 10% FBS and were incubated until a confluent monolayer was formed. Ten-fold serial dilutions of SFTSV in DMEM with 2% FBS were inoculated onto confluent cells. Following a 90 min incubation, DMEM with 2% FBS containing 0.5%, 1%, or 2% MC were overlaid and incubated at 37 °C with 5% CO₂. At 5 to 7 dpi, cells were fixed with 4% paraformaldehyde, and plaque formation was observed by staining the cells with 0.1% crystal violet. The virus titer was determined as plaque-forming unit per milliliter (PFU/mL).

4.3.5. Real-Time RT-PCR

Real-time RT-PCR was performed with the methods referred to our previous study (Hayasaka et al. 2015). Briefly, SFTSV viral RNA was extracted using ISOGEN-LS (NIPPON GENE Co., Ltd., Toyama, Japan). Primers of 965F (5'-GCRAGGAGCAACAARCAAACATC-3') and 1069R (5'-GCCTGAGTCGGTCTTGATGT C-3'), and probe FAM/5'-CTCCCRCCC-3'/ZEN/5'-TGGCTACCAAAGC-3' (Integrated DNA Technologies, Inc., Shinjuku, Japan) were used and real-time reactions were carried out using a TaKaRa One Step PrimeScript™ RT-PCR Perfect Real Time Kit (Takara Bio Inc., Shiga, Japan). Reactions were performed at 42 °C for 5 min, 40 cycles at 95 °C for 10 s, at 95 °C for 5 s, and at 60 °C for 34 s. The viral copy numbers were determined as the ratio of the copy numbers to the standard control prepared from an SFTSV gene-cloned plasmid vector (Hayasaka et al. 2015).

4.4. Results

4.4.1. CPE formation in SFTSV-infected cell lines

We first focused on CPE appearance in each cell line following SFTSV infection. TLT (tiger), FCWF-4 (cat), CRFK (cat), and CPK (pig) cells clearly showed CPE at 3, 5, 5, and 4 dpi, respectively (Figure 4.1. and Table 4.1). CPE features include cell rounding, aggregation, detachment, and shrinkage in size (Figure 4.1).

On the other hand, DFKT (wild deer), DFLT (wild deer), HHoVT (hedgehog), Vero E6 (monkey), BHK-21 (hamster), and A549 (human) cells did not exhibit apparent CPE after SFTSV infection (Figure 4.1.). These observations suggest that CPE formation due to SFTSV infection depends on cell species, and Felidae (cat- and tiger-derived) cells exhibited CPE.

4.4.2. Growth kinetics of SFTSV in cell lines

We next examined the growth kinetics of SFTSV in each cell line and determined the viral titers in the supernatants. In CPE-observed cell lines TLT, FCWF-4, CRFK, and CPK, viral titers increased until around 2 dpi and then were maintained or decreased (Figure 4.2A). Interestingly, TLT, FCWF-4, and CRFK showed higher virus yields in the supernatants, indicating $10^{3.3}$, $10^{3.1}$, and $10^{3.1}$ times increases at 2 dpi compared with those of 0 dpi, respectively (Table 4.1 and Figure 4.2A). CPK exhibited comparatively lower virus yield with $10^{1.4}$ times increase at 2 dpi compared with that of 0 dpi (Table 4.1 and Figure 4.2A). In these CPE-observed cells, viral titers of supernatants decreased after CPE-appeared days in TLT, FCWF-4, CRFK, and CPK cells at 3, 5, 5, and 4 dpi, respectively (Figure 4.2A). Also, the viral RNA levels were not significantly increased or decreased after CPE (Figure 4.2B), implying that viral yields did not occur after CPE in these cells.

In non-CPE cell lines of DFKT, DFLT, Vero E6, BHK-21, and A549, viral titers in the supernatants increased until 3–4 dpi (Figure 4.2A). Interestingly, viral titers in the supernatants varied among cell lines, showing $10^{1.4}$ to $10^{4.0}$ -fold increases at 4 dpi compared with those of 0 dpi (Table 4.1 and Figure 4.2A). In these cells, infectious viral titers in the supernatants were almost maintained or slightly decreased by 7 dpi (Figure 4.2A). On the other hand, viral RNA levels of Vero E6, BHK-21, DFLT, and DFKT continued to increase until around 6 dpi, whereas the levels of A549 decreased after 4 dpi (Figure 4.2B). This discrepancy might be due to offsetting titer decreases at 37 °C, because our unpublished results showed that infectious titers of SFTSV infectivity at 37 °C were gradually decreased (approximately $10^{0.2}$ FFU per day) (Nyampong et al. unpublished). Therefore, these observations suggest that viral propagation in these cells was maintained for 6–7 days.

On the other hand, viral titers of the supernatant in HHoVT cells did not increase over 0 dpi (Figure 4.2A). Viral RNA levels of HHoVT cells were slightly increased by 7 dpi; however, these levels were significantly lower than those of other cell lines (Figure 4.2B), suggesting that infectivity and viral propagation of SFTSV were very low in HHoVT cells.

4.4.3. PFA of SFTSV

We next performed PFA using CPE-observed cell lines TLT, CRFK, and FCWF-4. Interestingly, TLT exhibited clear plaque formation with 1% and 2% MC medium, but not 0.5% MC (Figure 4.3). On the other hand, FCWF-4 cells showed plaques, but the plaques were difficult to count due to variable sizes and non-round morphology (Figure 4.3). CRFK did not form apparent plaques under any MC condition (Figure 4.3). From

these results, TLT is likely to be useful for the PFA of SFTSV. Therefore, we compared the titers of SFTSV stock viruses (Table 4.2). Titers of PFU were approximately 2–3 times higher than FFU, but these differences were not significant as virus titers were in the log phase. These results indicate that TLT cells are useful for the titration of SFTSV by PFA depending on the experimental design.

4.5. Discussions

In this study, we evaluated the susceptibility to SFTSV of several mammalian cell lines, including our original cell lines. Tiger-derived TLT, cat-derived CRFK, and FCWF-4 cells exhibited apparent CPE and high viral yields following SFTSV infection. Pig-derived CPK cells also showed CPE, but viral yields were comparatively lower than those of TLT, CRFK, and FCWF-4 cells. Monkey-derived Vero E6, hamster-derived BHK-21, human-derived A549, and wild deer-derived DFKT and DFLT cells did not exhibit CPE. Among them, Vero E6 and BHK-21 showed comparatively higher viral yields than other cell lines. SFTSV infection in hedgehog-derived HHoVT cells was very limited. These observations suggest that different features, including CPE and virus yields, following SFTSV infection depend on the cell type. Our results provide useful information and tools for elucidating the mechanism of SFTSV infectivity, proliferation, and pathogenicity using in vitro models.

CPE induction in virus-infected cells is one of the keys to elucidating the pathogenic mechanism of infectious viruses. Previous studies have stated that viruses have complex life cycles in host cells and affect many aspects depending on the host's immune response and replication mechanisms of the virus (Turnell and Grand. 2012). Viral infection could cause damage to the cells and affect viral replication by hijacking their cellular processes for its own replication (McKinney et al. 2015). CPE due to viral infection is generally discerned through

cellular responses such as necrosis and apoptosis (Yang et al. 2022; Huang et al. 2023). Although SFTSV infection causes severe disease in humans and some kinds of animals, the type of cell death and the mechanism of CPE due to SFTSV are not fully elucidated. To elucidate SFTSV pathogenesis, it is important to provide a useful tool for the analysis of CPE in vitro. In this study, we identified four CPE-induced cell lines-TLT, CRFK, FCWF-4, and CPK-and these cell lines are expected to be useful to elucidate the mechanisms of CPE induced by SFTSV infection in vitro.

Our results indicated that the levels of viral propagation varied depending on cell type, including higher yields (TLT, CRFK, FCWF-4, Vero E6, and BHK-21) with more than about 10^3 -fold and lower yields (CPK, A549, DFLT and DFKT) with less than about 10^2 -fold increases in FFU compared with those of 0 dpi at around the peaks. High virus replication in Vero and BHK cells also aligns with previous findings (Tani et al. 2016). Interestingly, among higher yield cells, TLT, CRFK, and FCWF-4 cells were CPE-induced, while Vero E6 and BHK-21 did not exhibit CPE. In addition, CPK cells showed CPE, but viral yields were comparatively low, suggesting that CPE formation is not simply related to high viral propagation. Our findings provide useful information and tools to examine the mechanism of viral replication and proliferation of SFTSV in vitro by comparing cell lines that exhibit higher and lower virus propagation.

SFTS cases have been reported not only in humans but also in several animal species, including cheetahs, cats, and dogs (Kimura et al. 2018; Matsuno et al. 2018; Matsuu et al. 2019). In particular, there are many SFTS cat cases in Japan (Kida et al. 2019; Ando et al. 2021; Irie et al. 2022), and it seems that family *Felidae*, including cats, are susceptible to SFTSV infection and can result in severe disease, because fatality of SFTS in cats was up to

60% and two fatal cases of cheetah were reported (Matsuu et al. 2019; Matsuno et al. 2018; Ando et al. 2021). Previous studies of in-vivo experiments and positive cases in cat species revealed severe lesions in the spleen, lymph nodes, and gastrointestinal tract, suggesting a significant impact of SFTSV on felines (Matsuno et al. 2018; Park et al. 2019a; Ando et al. 2021). For one of the mechanisms of SFTSV pathogenicity in mammalian cells, the anti-STAT2 activity of SFTSV has been shown to determine species-specific pathogenicity in hosts cell (Yoshikawa et al. 2019). STAT2 is involved in type I and type III interferon signaling, which are important for the immune response against viral infection (Ivashkiv and Donlin. 2014; Duncan and Hambleton. 2021). The inability of SFTSV NSs protein to bind to STAT1 and STAT2 of murine cells makes murine cells permissive to SFTSV infection (Yoshikawa et al. 2019), and the mechanism involving STAT1 in humans and cats during SFTSV infection may be different and might affect the pathogenicity of SFTSV (Yoshikawa et al. 2023).

Therefore, cell lines including cats and other *Felidae* are required as tools for further analyses of SFTSV infection in vitro. In this study, tiger-derived TLT, cat-derived CRFK and FCWF-4 cells were very susceptible to SFTSV infection with high viral propagation and CPE appearance, suggesting that these cells are useful to further elucidate the high pathogenicity of SFTSV in cats and related animals. In particular, our original cell line TLT showed high viral propagation and induced apparent CPE, including clear plaque formation; thus, further studies using this TLT cell line will provide useful clues to elucidate the mechanism of SFTSV infectivity and pathogenicity.

SFTSV infects multiple kinds of animals, including wildlife and livestock (Tabara et al. 2016; Yu et al. 2018), although those animals are unlikely to develop diseases (Liang et al. 2014; Chen et al. 2019). For example, SFTSV surveillance on wild deer in Japan conducted

between 2010 and 2020 showed that 55% of deer tested positive for anti-SFTSV antibodies, and 25% during 2013–2014, indicating notable seroprevalence among wild deer (Kimura et al. 2018; Hashimoto et al. 2022b). A study conducted in Korea detected SFTSV viral RNA in water deer tissues, one of the most common wild ungulates in Korea, thus raising concern about transmission between humans and wildlife (Lee et al. 2020a). In this study, we showed the susceptibility to SFTSV of our original wild deer-derived cells DFKT and DFLT, and these cell lines may be useful models to examine SFTSV infectivity and pathogenicity in deer.

European hedgehogs are suggested to be reservoirs of some viral diseases such as tick-borne encephalitis virus (TBEV) and coronaviruses (Saldanha et al. 2019; Schönbächler et al. 2019; Domanska-Blicharz et al. 2024). However, our original hedgehog-derived HHoVT cells did not show apparent viral infectivity or propagation. In preliminary experiments, HHoVT cells did not exhibit susceptibility to SFTSV infection even at higher MOI (0.1 and 1). From the current data, we cannot conclude the factors of non-infectivity of SFTSV in hedgehogs; however, it seems important to elucidate the mechanism of inability of infection in this mammalian cell. Although SFTSV infectivity in HHoVT cells was limited, this cell line seems to be useful to determine a key of SFTSV infectivity in mammalian cells, focusing on receptors or other cell factors by specific gene knockout or overexpression experiments.

For titration of infectious SFTSV, immunostaining methods such as FFA are required, because SFTSV infection does not induce CPE in most cell lines (Yu et al. 2011; Sun et al. 2018; Yoshikawa et al. 2023). However, FFA is generally time-consuming and costly compared with PFA. Taniguchi et al. 2017 reported a PFA method for neutralization assay using highly passaged SFTSV strain; however, the cell lines utilized for PFA for SFTSV have been limited. In this study, we successfully achieved plaque formation using TLT and FCWF-

4 cells, and TLT showed clear plaques that were easy to count. We used medium including methylcellulose to make foci, and methylcellulose offers better safety characteristics and handling convenience within containment compared to agar, even though agar provides proven performance and cost-effectiveness (Fenner et al. 2014). The titers of PFU by PFA were not significantly different compared with those of FFU by FFA in log phase. Our results provide useful information and a cell line for the PFA for SFTSV.

4.6. Conclusions

This study presents a comprehensive analysis of cell line susceptibility to SFTSV infection, and our findings will provide useful information and tools for further elucidation of the mechanisms of SFTSV infectivity, proliferation, and pathogenicity using in vitro models. In particular, our original tiger-derived TLT cell line is expected to support further analyses of the SFTSV high pathogenicity in vitro and serves as a useful cell for PFA. Although SFTSV susceptibility in our original cell line of hedgehog-derived HHoVT cell line was limited, this cell line is expected to be useful for determining the factors of SFTSV infectivity as a negative control compared with susceptible mammalian cells. Our results will contribute to further studies of SFTSV, including elucidation of the mechanisms of infectivity and pathogenicity in vitro, as well as for the development of improved diagnostic and research tools and the development of strategies to combat this emerging viral threat.

4.7. Tables of Chapter 4.

Table 4.1. Cytopathic effect formation in SFTSV-infected cell lines.

Origin				CPE	Viral Titers (FFU/mL)	
Species		Tissue		Observed		
CPE cells				dpi	0 dpi	2 dpi
TLT	Tiger	Liver	established	3	10 ^{3.8}	10 ^{7.1}
FCWF-4	Cat	Macrophage	commercial	5	10 ^{3.4}	10 ^{6.5}
CRFK	Cat	Kidney	commercial	5	10 ^{3.4}	10 ^{6.5}
CPK	Pig	Kidney	commercial	4	10 ^{3.8}	10 ^{5.2}
Non-CPE cells					0 dpi	4 dpi
DFKT	Wild deer	Kidney	established	-	10 ^{3.4}	10 ^{5.2}
DFLT	Wild deer	Liver	established	-	10 ^{3.4}	10 ^{5.6}
HHoVT	Hedgehog	Ovary	established	-	10 ^{3.4}	-
Vero E6	Monkey	Kidney	commercial	-	10 ^{3.4}	10 ^{6.7}
BHK-21	Hamster	Kidney	commercial	-	10 ^{3.4}	10 ^{6.4}
A549	Human	Lung	commercial	-	10 ^{3.4}	10 ^{5.0}

Table 4.2. Comparison of SFTSV viral titration of FFA and PFA.

SFTSV Strain	FFA (FFU/mL)	PFA (PFU/mL)
	Vero E6	TLT
Tk-F123	4.3×10^7	7.3×10^7
Ng-F264	4.8×10^7	1.3×10^8

4.8. Figures of Chapter 4.

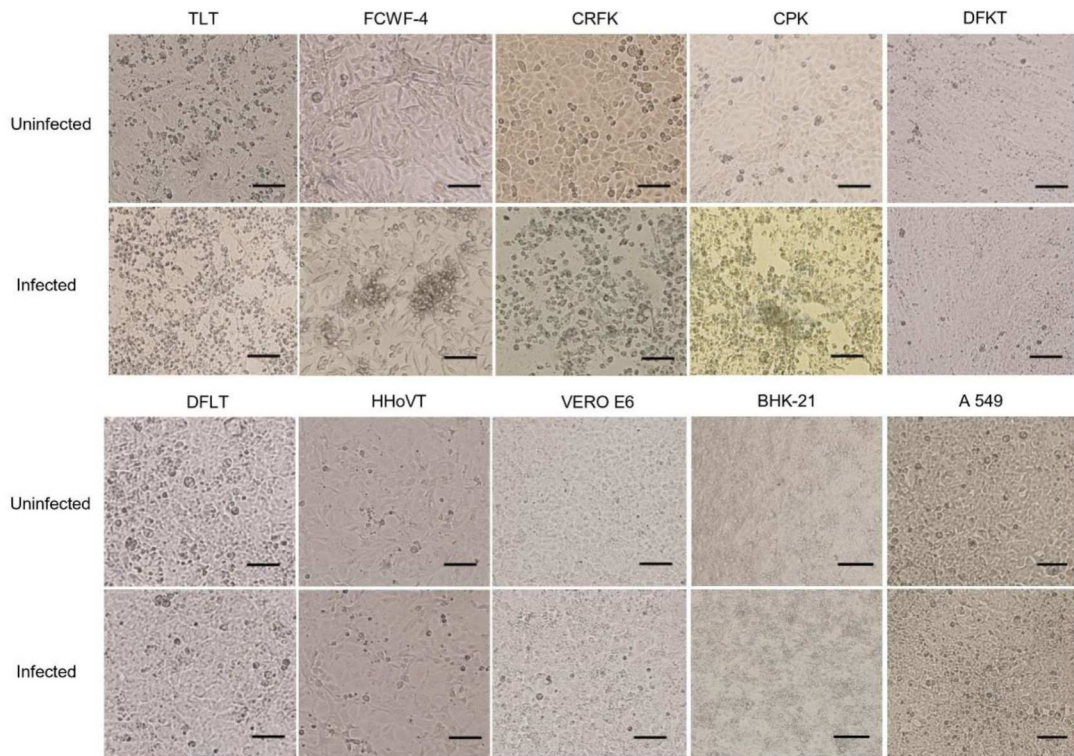


Figure 4.1. CPE formation in different cell lines following SFTSV infection. Each cell line was infected with SFTSV at an MOI of 0.01 and microscopic images of TLT (3 dpi), FCWF-4 (5 dpi), CRFK (5 dpi), CPK (4 dpi), DFKT (5 dpi), DFLT (5 dpi), HHoVT (5 dpi), Vero E6 (5 dpi), BHK-21 (5 dpi), and A549 (5 dpi) are shown. SFTSV-infected TLT, FCWF-4, CRFK, and CPK cells exhibit apparent CPE compared with uninfected controls. Morphologies of SFTSV-infected DFKT, DFLT, HHoVT, Vero E6, BHK-21, and A549 cells are similar to those of uninfected controls. Scale bars indicate 100 μ m.

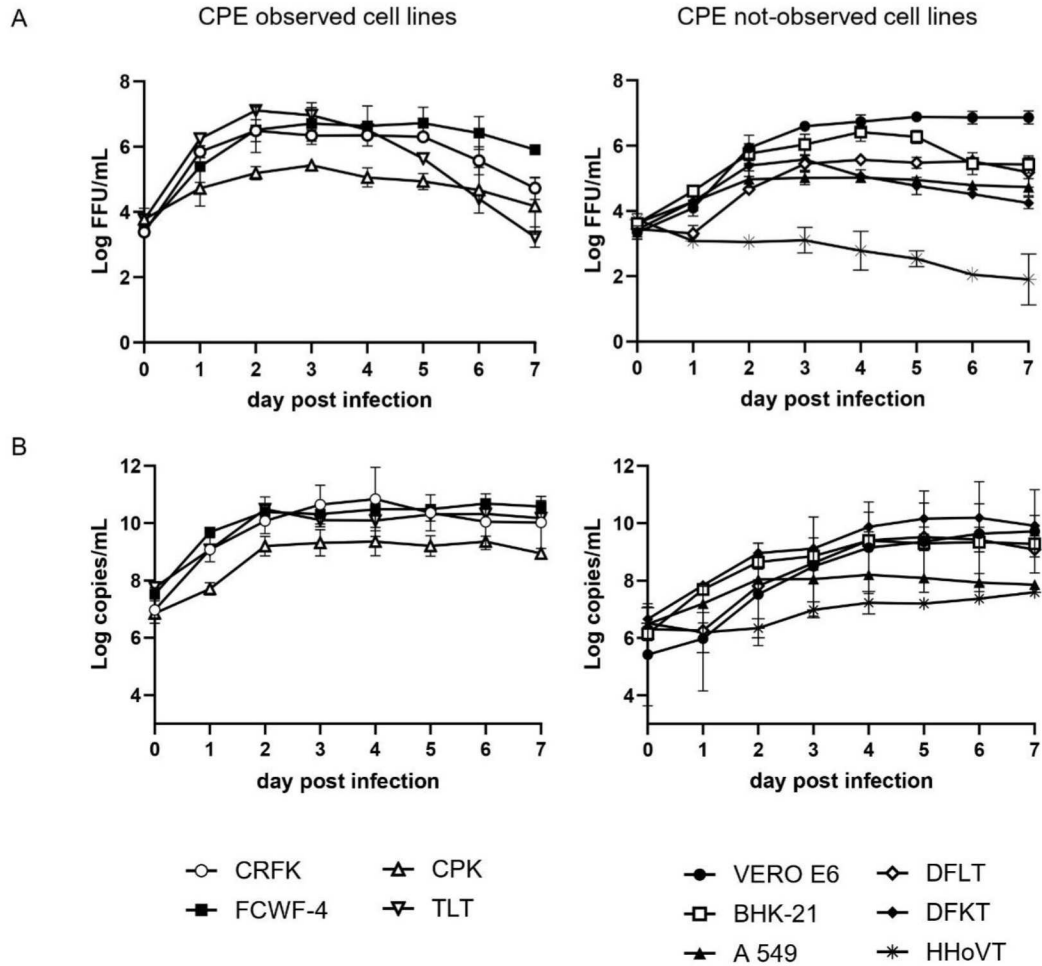


Figure 4.2. Growth kinetics of SFTSV in different cell lines. (A) Infectious virus titers and (B) viral copy numbers in the cultured supernatants of each cell. Each cell line was infected with SFTSV at an MOI of 0.01, and the supernatants were collected daily. Data are presented as the mean $\pm$ standard deviation from three independent experiments.

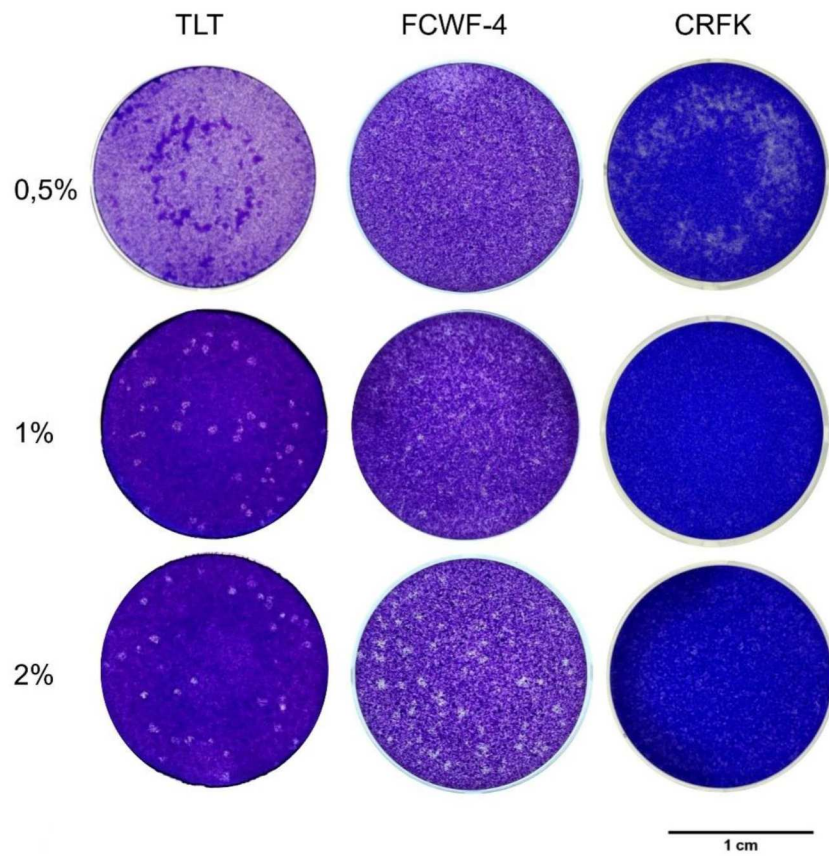


Figure 4.3. Plaque formation of SFTSV-infected TLT, FCWF-4, and CRFK cells. After inoculation with SFTSV, cells were overlaid with medium containing 0.5%, 1%, or 2% MC. The plaques were observed after 5 days in TLT cells and 7 days on FCWF-4 cells. FCWF-4 cells exhibited limited visibility of plaques compared with TLT cells. CRFK cells showed no visible plaques. Plaque morphologies with 1% MC were clear, whereas 2% MC reduced plaque size and visibility.

5. GENERAL CONCLUSION

This study addressed two gaps in SFTSV research by developing a practical diagnostic tool and expanding basic virological knowledge. The dried RT-LAMP assay provides an alternative diagnostic method for SFTSV detection using serum and plasma samples, offering a practical point-of-care testing (POCT) solution that only requires basic equipment such as heat block, portable LED illuminator, and pre-loaded LAMP tubes containing dried reagents with convenient storage. This cost-effective, equipment-free approach overcomes diagnostic accessibility barriers in resource-limited settings where commercial kits and advanced laboratory facilities are unavailable. It enables rapid diagnostic results in rural, endemic areas, although confirmatory testing remains recommended. Future research including expanding RT-LAMP assay validation across diverse host samples and conducting field trials in an endemic region to assess the diagnostic performance should be done.

Additionally, our comprehensive evaluation of susceptibility of mammalian cells to SFTSV infection provides insight into advancing fundamental virology research. Notably, our original tiger-derived TLT cell line showed outstanding potential for SFTSV studies, supporting high viral replication and plaque formation essential for plaque-forming assays (PFA), while our hedgehog-derived HHoVT cell line, despite its restricted susceptibility, serves as an important negative control for investigating mechanism regulating SFTSV infectivity. Investigating the molecular determinants of cellular mechanisms during SFTSV infection should be further elucidated.

These findings provide both immediate practical SFTSV diagnostic solution for rural areas and research platforms for studying viral infectivity, replication, and pathogenesis of SFTSV *in vitro*.

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