

SCREENING OF ZONOTIC HOSTS OF SEVERE FEVER THROMBOCYTOPENIA SYNDROME VIRUS VIA NESTED RT-PCR IN CHIANG MAI, THAILAND

Saralee Srivorakul¹, Kidsadagon Pringproa^{1,2}, Wei-Li HSU³, Chatchote Thitaram^{1,2}, and Thunyamas Guntawang^{1,2*}

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Abstract

Severe Fever Thrombocytopenia Syndrome Virus (SFTSV) is primarily transmitted by tick vectors and is capable of infecting both humans and animals across various countries, particularly in Asia. The first human case in Thailand was detected in 2019 in the central region, with subsequent reports of animal infections in the central and northeastern regions. This study aimed to investigate animals in Chiang Mai, located in northern Thailand, for SFTSV infection. Serum samples were obtained from 422 animals, comprising elephants, cats, dogs and others, and screened using RT-PCR. All samples tested negative for SFTSV. These findings provide significant data for future surveillance and underscore the necessity of ongoing monitoring for suspected zoonotic transmission.

Keywords: Animals; RT-PCR; Screening; SFTSV

Introduction

Emerging tick-borne zoonotic disease Severe Fever Thrombocytopenia Syndrome Virus (SFTSV) raises serious public health issues all throughout Asia. Although it was initially recognized for its severe impact on humans, recent research has emphasized its presence and potential effects in various animal populations. SFTSV is classified as a Bunyavirus, ss-RNA, comprising 3 main segments consisting

of Large (L), Medium (M) and Small (S). RNA-dependent RNA polymerase (RaRp) is encoded by the L segment (~6,368 bases), whereas the viral envelope glycoproteins G1 and G2, which are necessary for immune recognition, are encoded by the M segment (~3,378 bases). The nonstructural protein (NSs) and nucleocapsid protein (NP) are encoded by the S segment (~1,744 bases)

¹ Faculty of Veterinary Medicine, Chiang Mai University, Thailand, 50100. E-mail: ann_nice@hotmail.com, ORCID: 0009-0003-0258-4273; kidsadagon.p@cmu.ac.th, ORCID: 0000-0001-7291-1100; chatchote.thitaram@cmu.ac.th, ORCID: 0000-0001-9319-5596; thunyamas.g@cmu.ac.th*, ORCID: 0009-0005-5011-0421

² Center of Elephant and Wildlife Health, Chiang Mai University Animal Hospital, Chiang Mai, Thailand, 50100. E-mail: kidsadagon.p@cmu.ac.th, ORCID: 0000-0001-7291-1100; chatchote.thitaram@cmu.ac.th, ORCID: 0000-0001-9319-5596; thunyamas.g@cmu.ac.th*, ORCID: 0009-0005-5011-0421

³ Graduate Institute of Microbiology and Public Health, National Chung Hsing University, Taichung, Taiwan, 40227. E-mail: wlhsu@dragon.nchu.edu.tw, ORCID: 0000-0002-1392-163X

* Corresponding author

(Wang *et al.*, 1993; Xu *et al.*, 2011; Zhang *et al.*, 2021). SFTSV is spread by *Haemaphysalis longicornis* ticks, mosquitoes and sandflies. (Xu *et al.*, 2011; Yun *et al.*, 2014; Luo *et al.*, 2015).

Three stages of SFTSV infection manifest clinically, starting with the fever stage, in which patients have a high fever, leukopenia, headache, and diarrhea in addition to laboratory results of thrombocytopenia. Multiple organ failures, internal bleeding, disseminated intravascular coagulation (DIC), and neurological symptoms occur frequently in the second stage. If patients recover, laboratory values will gradually normalize. The mortality rate is estimated at 5.3% (Deng *et al.*, 2013; Liu *et al.*, 2014; Zhan *et al.*, 2017). South Korea, Japan, Vietnam, Myanmar and Thailand are among the Asian countries where confirmed cases have been reported (Kim *et al.*, 2013; Takahashi *et al.*, 2014; Tran *et al.*, 2019; Ongkittikul *et al.*, 2020; Win *et al.*, 2020).

Over 40% of animal samples in China, both domestic and wild populations, demonstrated antibodies to the virus, with viral RNA detected in approximately 17% of cases (Huang *et al.*, 2019). Taiwan also reported a high prevalence of viral RNA (over 60%) in asymptomatic ruminants, including cattle, goats and sheep, suggesting these animals could act as reservoirs (Kuan *et al.*, 2023). The severity of SFTSV in animals was further studied by experimental animals, where two out of three cats infected with the virus succumbed to the disease, exhibiting more severe symptoms than observed in humans, including high fever, marked leukopenia and thrombocytopenia, weight loss, lethargy, jaundice, severe gastrointestinal hemorrhage and significant lesions in lymph nodes and spleen (Park *et al.*, 2019). In Thailand, evidence of SFTSV infection in animals has also emerged, with reports in cats (Ongkittikul *et al.*, 2020), dogs (Ishijima *et al.*, 2023) and wild rodents (Linsuwanon *et al.*, 2024), primarily concentrated in the central region of the country.

Asian elephants are an endangered species at risk for Elephant Endotheliotropic Herpesvirus (EEHV), a fatal hemorrhagic disease (Long *et al.*, 2016; Guntawang *et al.*, 2021). Nonetheless, certain elephants exhibited clinical manifestations similar to SFTSV, including severe hemorrhage and thrombocytopenia, despite showing negative results for EEHV. This observation suggests the potential of SFTSV as an alternative pathogen. This research aims to investigate SFTSV RNA in samples from elephants, companion animals (dogs and cats) and other species in Chiang Mai, Northern Thailand, to address the knowledge gap and enhance our understanding of SFTSV epidemiology.

Materials and Methods

Sample Collection and RNA Extraction

The study collected 422 serum samples, including elephants, cats, dogs, ruminants and others between June, 2023, and March, 2025. Ethical approval was exempted, as the study used only archival animal serum collected for diagnostic purposes during routine veterinary procedures. These samples were obtained by veterinary staff at a small animal hospital within the Faculty of Veterinary Medicine and the Center of Veterinary Medical Diagnostic and Animal Health Innovation at Chiang Mai University. The elephant samples were specially shared by the Elephant Health Monitoring Program. The RNA was then extracted using a commercial kit (QIAamp® Viral RNA Mini kit, Qiagen, USA), and quantified with a Nanodrop spectrophotometer (NanoDrop One, Thermo Fisher Scientific™, USA). The RNA templates were then converted to cDNA using a Revertra Ace™ qPCR RT Master Mix (Toyobo, Japan) according to the manufacturer's instructions, then stored at -20°C for further use.

Construction of plasmid for positive control

The partial of SFTSV S segment (Accession No. AB817995), position 44 to 781, was cloned into pET24a+ plasmid and transformed into *Escherichia coli* strain DH-5α. Genomic DNA was then extracted from the plasmid for use as a positive control for this study.

Nested RT-PCR

The SFTSV-screening nested RT-PCR amplification was performed using QuickTaq HS Dye Mix (Toyobo, Japan) in 20 µL reaction volume, consisting of 10 µL of 2× QuickTaq Master Mix, 0.4 µL each of 10 µM forward (5'-CATCATTGTCTTTGCCCTGA-3') and reverse primers (5'-AGAAGAC AGAGTTCACAGCA-3'), 200 ng of cDNA or plasmid template, and nuclease-free water to the final volume (Yoshikawa *et al.*, 2014; Kuan *et al.*, 2023). To confirm the result by nested RT-PCR, 2 µL of the positive initial PCR amplicon was utilized as the template for the second run, utilizing inner set primers (Forward: 5'-AAYAAGATCGTCAAGGCATCA-3'; Reverse: 5'-TAGTCTTGGTGAAGGCATCT T-3') (Kuan *et al.*, 2023). The thermal cycling conditions were as follows: initial denaturation at 95°C for 2 min and 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 68°C for 30 sec; followed by a final extension at 68°C for 5 min. PCR products were analyzed by

electrophoresis on a 1.2% (w/v) agarose gel in TAE buffer to verify amplicon size.

Results and Discussion

The results of RT-PCR were performed in 422 animal serum samples, including 198 Asian elephants, 69 cats, 118 dogs and 37 other animal species, including 14 cattle (*Bos taurus*), 7 chickens (*Gallus gallus domesticus*), 4 goats (*Capra hircus*), 3 Buffaloes (*Bubalus bubalis*), 2 ducks (*Anas platyrhynchos*), 2 horses (*Equus caballus*), 1 eland (*Taurotragus oryx*) and 1 fallow deer (*Dama dama*). All animals were reported as negative results for SFTSV on the first round of RT-PCR (Table 1).

Quality control of nested RT-PCR was performed using positive control from the constructed plasmid, showing the size as expected (Figure 1).

This study is the first screening in the upper north of Thailand; our recent screening of 422 animals in Chiang Mai, using nested RT-PCR, yielded no positive cases. This suggests a low or absent prevalence of SFTSV in the local animal population, although continuous surveillance is essential. Based on all previous reports, SFTSV has been detected in both animals and humans primarily in central Thailand, with a few rare cases reported in the northeast and north of the country (identified through either DNA analysis or seroprevalence studies). All SFTSV prevalence timelines were summary described below in Figure 2.

The occurrence of SFTSV in Thailand has been reported and shown in Figure 2 with scattered red area Nos. 1-6. Red area No. 1, the earliest report, dates back to 2019 in central Thailand, where a 70-year-old woman was infected after exposure to her cats, which had died prior to her illness. Notably, her cats tested positive for SFTSV, while her contacted people nearby showed negative PCR results, although positive ELISA titer levels exceeded 100, indicating previous exposure (Ongkittikul *et al.*, 2020). After the first report, further investigations were carried out and are demonstrated in red area No. 2, which involved screening 8 contacted humans, 4 dogs, 3 cats and a rat around the case. All humans and animals tested PCR-negative; however, serological testing revealed that all dogs and cats had SFTSV-specific antibodies (ELISA), suggesting prior exposure (Sansilapin *et al.*, 2024). For red area No. 3, SFTSV observation in human populations was performed by screening 712 serum samples at Chulalongkorn Hospital, identifying 3 positive cases via RT-qPCR, 2 from Bangkok and 1 from Chachoengsao, indicating confirmed active infections (Rattanakomol *et al.*, 2022). In animals,

Table 1. RT-PCR results of SFTSV detection in animal samples in Chiang Mai, Thailand, between June, 2023, and March 2025

Animal	N	RT-PCR Result	
		Positive	Negative
Elephants	198	0	198
Cats	69	0	69
Dogs	118	0	118
Others	37	0	37
Total	422	0	422

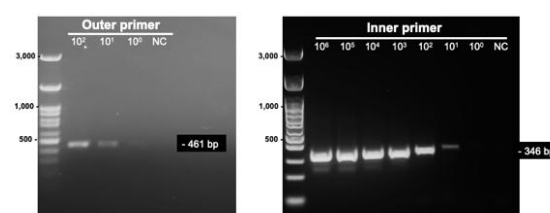


Figure 1. Nested RT-PCR of serially diluted SFTSV plasmid. The amplification was performed with 2 primer sets. NC = negative control

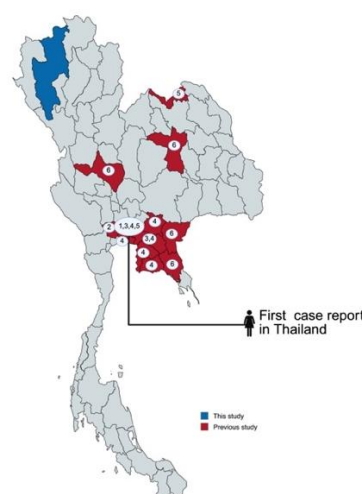


Figure 2 The incidence of SFTSV from human and animal reports in Thailand during 2019-2024 based on molecular detection or seroprevalence studies. The red areas indicated locations where SFTSV cases have been previously detected, while the blue area represents no confirmed cases. The numbers are defined in the Discussion section. The map was created using MapChart.net.

as illustrated in red area No. 4, except for the initially infected cat in 2019, a broader screening of 57 dogs found 1 serum sample positive for SFTSV via RT-PCR. Additionally, serology conducted on

455 samples across several provinces found 106 animals detected with SFTSV antibody titers ranging from Chonburi with 70 cases, to Samutprakarn, Bangkok, Chachoengsao, Rayong and Prachinburi, in descending order (Ishijima *et al.*, 2023). Moreover, SFTSV seroprevalence investigation explored antibodies in 7 humans from the northeast (Nongkhai), indicating prior exposure (Saba Villarreal *et al.*, 2024), as shown in red area No. 5. Furthermore, wild rodent populations examined between 2019 and 2023 involving 1,019 samples across multiple regions, as shown in red area No. 6, revealed 11 positive rodent samples found in Nakornsawan, Chanthaburi, Srakaew, and Khonkaen (approximate positivity rate of 1.1%), supporting the presence of SFTSV in wildlife reservoirs (Linsuwanon *et al.*, 2024).

While a truly negative result for SFTSV detection by RT-PCR is possible, several factors may contribute to false-negative results. These include the adequacy of sample collection, RNA extraction quality, PCR assay sensitivity, potential genetic variation in the target region, and other technical errors. Regarding sample collection, SFTSV is primarily detected in serum, which is commonly used for PCR detection or ELISA seroprevalence studies. In our protocol, sera were immediately refrigerated following extraction to preserve RNA integrity. Additionally, other clinical specimens, such as throat swabs, cerebrospinal fluid and urine, have been reported as viable sources for SFTSV genomic detection (Yoshikawa *et al.*, 2014). In this study, RNA concentration was quantified using a Nanodrop spectrophotometer, and subsequently converted to cDNA. The RT-PCR assay employed demonstrated a limit of detection of 10 copies/ μ L (Figure 1, lane 2), which is adequate for early-stage detection. The assay targeted the S segment of the viral genome, which prior studies have identified as more sensitive for SFTSV detection compared with the M or L segments. The primers used in this study were designed to bind conserved regions shared by both the Japanese strain HB29 and the Chinese strain YG1 (Yoshikawa *et al.*, 2014). Genetic analyses of SFTSV strains circulating in Thailand revealed a 99.7% similarity to Chinese strains (Rattanakomol *et al.*, 2022) and a close relation to Japanese strains (Rattanakomol *et al.*, 2022; Ishijima *et al.*, 2023). This study used nested RT-PCR because it detected the target for a longer period after sample collection. In contrast, qPCR is most effective within the first 7 days, but its detection rate decreases for samples collected between days 8 and 21 (Jalal *et al.*, 2021). Future screening should consider targeting animals exhibiting clinical signs consistent with SFTSV infection or conducting surveillance in animals with

close contact to tick vectors, which serve as reservoirs for SFTSV transmission (Luo *et al.*, 2015). This study provides a basis on molecular detection. However, a key limitation is the absence of serological data. To build upon this work, future investigations should be integrated serological methods, approaching the essential needs for advancing both clinical diagnostics and public health screening strategies

Conclusions

SFTSV has been identified in both animal and human populations, mainly in central Thailand, with some cases reported in the northeastern regions. While there have been negative results in Chiang Mai, continued surveillance and research are essential to fully understand the distribution and zoonotic transmission dynamics of SFTSV across Thailand.

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Authors' contributions

Srivorakul, S.: Sample collection; Guntawang, T.: Writing original draft, Methodology, Investigation, Conceptualization, Funding acquisition; Pringproa, K.: Conceptualization, Investigation; Thitaram, C.: Investigation; Hsu, W.-L.: Investigation, consultant. All authors have read and agreed to the published version of the manuscript.

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