Development of a real-time loop-mediated isothermal amplification method for the detection of severe fever with thrombocytopenia syndrome virus

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Severe fever with thrombocytopenia syndrome (SFTS) is being reported annually in South Korea since its first detection there in 2010. The causal agent is a negative-strand RNA virus 80–100 nm in diameter. It causes fever, thrombocytopenia, leukocytopenia, gastrointestinal symptoms, and neural symptoms. The mortality rate of SFTS was 32.6% among 172 cases reported from 2012 to 2015 in South Korea. Thus, is necessary to develop an effective diagnostic method that selectively identifies the isolates circulating in South Korea. The real-time reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay is a simple, rapid, and sensitive approach for molecular diagnosis. Here, we designed novel primers for this assay and found that the technique had very high specificity, sensitivity, and efficiency. This real-time RT-LAMP approach using the novel primers developed herein can be applied for early diagnosis of SFTSV strains in South Korea to reduce the mortality rate of SFTS.

Keywords: severe fever with thrombocytopenia syndrome (STFS), reverse transcription loop-mediated isothermal amplification (LAMP), molecular diagnostics, virus detection

Introduction

Since 2009, cases of acute febrile illness in the form of severe fever with thrombocytopenia syndrome (SFTS) have been reported in China (Yu *et al.*, 2011). A new virus was isolated from these patients, which was identified to be SFTS bunyavirus (Yu *et al.*, 2011). RNA sequence analysis showed that the virus belongs to a member of the *Phlebovirus* genus in the *Bunyaviridae* family (Yu *et al.*, 2011). However, according to the nomenclature by the International Committee of Taxonomy of Viruses (ICTV), SFTS virus (SFTSV) has been classified into the genus *Banyangvirus*, family *Phenuiviridae* (Takayama-Ito and Saijo, 2020). Surveillance data indicate that the incidence of SFTS is increasing throughout Asia, especially in China and Korea (Choi *et al.*, 2016; Park *et al.*, 2016; Wu *et al.*, 2016; Zhang *et al.*, 2017). The populations at the highest risk are farmers and elderly women living in rural farming communities and wooded and hilly areas, where immediate health care might not be available. In addition, human-to-human transmission of the virus through blood or close nosocomial contact has been reported (Xiong *et al.*, 2012). Similar to other members of the family *Phenuiviridae*, SFTSV is a negative-strand RNA virus, and it comprises three single-stranded RNA genome segments designated as large (L), medium (M), and small (S) (Jiao *et al.*, 2013). SFTSV is a spherical virion 80–100 nm in diameter, covered by a 5–7-nm-thick lipid bilayer envelope (Li, 2011; Lu *et al.*, 2011; Yu *et al.*, 2011; Xiong *et al.*, 2012). SFTSV infection typically has four distinct phases: incubation, fever, multiple organ failure, and convalescence (Liu *et al.*, 2014). SFTS patients exhibit fever, thrombocytopenia, leukocytopenia, gastrointestinal and neural symptoms, and bleeding tendency. The levels of liver and cardiac enzymes are commonly increased in SFTS patients, followed by damage in the liver and heart, respectively (Yu *et al.*, 2011; Gai *et al.*, 2012). SFTS was first reported in China in 2009, followed by Korea in 2012, and Japan in 2013. However, evidence of SFTS infections in South Korea from 2010 has been found, indicating that SFTS infections in South Korea occurred around the same time as reported in China (Kim *et al.*, 2018). A total of 172 cases were reported in South Korea from 2012 to 2015, with a mortality rate of 32.6% (Choi *et al.*, 2016). This mortality rate is higher than that recently reported in China (12.2%); the first country where SFTS was reported (Guo *et al.*, 2016). For early detection and diagnosis, quantitative real-time reverse transcription PCR (RT-PCR) and double-antigen sandwich enzyme-linked immunosorbent assay (ELISA) have been used thus far. These methods detect a specific genetic region, which is the S segment, because the S segment is the most conserved gene. However, the L segment also has a conserved region and can be used as a target for the loop-mediated isothermal amplification (LAMP) method. There are no differences in sensitivity or specificity by the L, M, and S segments (Jiao *et al.*, 2012; Sun *et al.*, 2012; Yoshikawa *et al.*, 2014; Baek *et al.*, 2018). The RT-PCR and ELISA methods have limitations compared with the LAMP method, which is simpler and more rapid and sensitive for *in vitro* nucleic acid amplifica-

tion (Yang *et al.*, 2012; Huang *et al.*, 2014). LAMP can detect very few copies of DNA under isothermal conditions. It is highly efficient, sensitive, and specific for target sequences. Moreover, LAMP is applicable to RNA upon use of reverse transcriptase and DNA polymerase (Notomi *et al.*, 2000). Because of these advantages of the LAMP method, RT-LAMP has already been applied in the detection of several RNA viruses, such as enterovirus 71, influenza A H1N1 virus, coronavirus, West Nile virus (WNV), and Ebola virus (Parida *et al.*, 2004, 2011; Poon *et al.*, 2005; Kurosaki *et al.*, 2007; Ma *et al.*, 2010; Jiang *et al.*, 2011). In this study, we applied newly designed LAMP primers to target the L genes of SFTSV isolated in South Korea, and we determined the specificity, sensitivity, and efficiency of this approach.

Materials and Methods

Virus samples and RNA extraction

Jeju Halla General Hospital received written consent for sample collection from adult patients with SFTSV infection. The Korea Centers for Disease Control and Prevention (KCDC) standard protocol (conventional PCR) was used for detecting and confirming the virus as SFTSV. The Institutional Review Board reviewed and approved the use of the study samples for the purpose of research, as this study does not affect patients. All the experimental work and sample collections were supervised by the Catholic Medical Center Office of the Human Research Protection Program (CMC OHRP) of South Korea (approval number MC19SESI0015). The SFTSV CUK-JJ01 strain propagation, cell lines maintenances, and viral RNA extraction were performed as described in a published study (Won *et al.*, 2019). *In vitro* experiments using SFTSV were conducted in a Bio Safety Level 3 (BSL-3) facility at the Korea Zoonosis Research Institute, Jeonbuk National University. The control RNA viruses were WNV, Japanese encephalitis virus (JEV), dengue viruses 2 and 3 (DENV-2, DENV-3), Zika virus, chikungunya virus, and influenza virus (H5N1). Their RNAs were extracted from 140 μl of the viral stocks using QIAamp® Viral RNA Mini Kits (Qiagen, #52906) according to the manufacturer's instructions.

Design of primers for real-time RT-LAMP

A total of 44 genomic sequences of different strains of SFTSV were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and analyzed using CLC Main Workbench 7 (version 7.6.4.) to identify conserved regions of the nucleotide sequence of SFTSV. Six SFTSV-specific RT-LAMP primers targeting the L gene were designed (Table 1). The primers consist of two external primers (forward outer primer F3 and backward outer primer B3), two internal primers (forward inner primer FIP and backward inner primer BIP), and two loop primers (forward loop primer LoopF and backward loop primer LoopB). The reaction was carried out at 65°C for 50 min using a WarmStart LAMP Kit (NEB, #E1700).

Conventional RT-PCR and real-time RT-PCR primer design and amplification

The Qiagen One-Step RT-PCR Kit (Qiagen, #210212) was used for conventional RT-PCR, using the primers NP2-F (5′-CATCATTGTCTTTGCCCTGA-3′) and NP2-R (5′-AGAAGACAGAGTTCACAGCA-3′) (Oh *et al.*, 2016). Reverse transcription reactions were incubated at 50°C for 30 min, followed by 95°C for 15 min. The amplification was performed according to the following conditions: 30 sec at 94°C, 30 sec at 52°C, and 1 min at 72°C for 35 cycles, followed by a final extension step at 72°C for 10 min. The iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad, #172-5150) was used for real-time RT-PCR. The primers 5′-GGGTCCCTGAAGGAGTTGTAAA-3′ and 5′-TGCCTTCACCAAGACTATCAATGT-3′ were designed to target conserved sequences in the S segment of this virus (Cui *et al.*, 2012). Real-time RT-PCR cycling was performed on a Bio-Rad® CFX96™ system as follows: reverse transcription reaction at 50°C for 10 min, polymerase activation at 95°C for 1 min, and amplification under 40 cycles of denaturation at 95°C for 10 sec and annealing/extension at 60°C for 30 sec.

Synthesis of RNA transcripts for testing the sensitivity and efficiency

Synthesis of RNA transcripts and calculation of copy numbers were performed according to previous studies (Devonshire *et al.*, 2011; Lee *et al.*, 2011). RNA transcripts were prepared for three types of assays: conventional RT-PCR, real-time RT-PCR, and real-time RT-LAMP, each containing the necessary primer sequence information. Serial 10-fold dilutions of RNA transcripts with titers from 5×10^9 copies to 5×10^1 copies were used as templates. We evaluated the sensitivity and efficiency of the three assays.

Results

Specificity of the real-time RT-LAMP assay

For the specificity test of the real-time RT-LAMP assay, we used extracted viral RNA as the template. The results showed

Table 1. Primer design

Primer	Sequence (5′-3′)	Position[a]
F3	TGGTACATTGATGCTGTGG	2678–2696
B3	TCCACTTCTCGGCATCA	2968–2984
FIP	CTCTGAGGCCACCATGCTGGAAGGCAGTTCTAGATGACG	2758–2778, 2709–2726
BIP	CCACACGAGACTGTTGCCAGGCTGACTTCAGTCCATG	2858–2876, 2909–2926
LoopF	TTGAAGAGACAGATTCGCATG	2731–2751
LoopB	CTAGGCTCAAGAATTCCATCATAGA	2880–2904

[a] Genome position according to the strain's complete sequence (GenBank accession number MH937374).
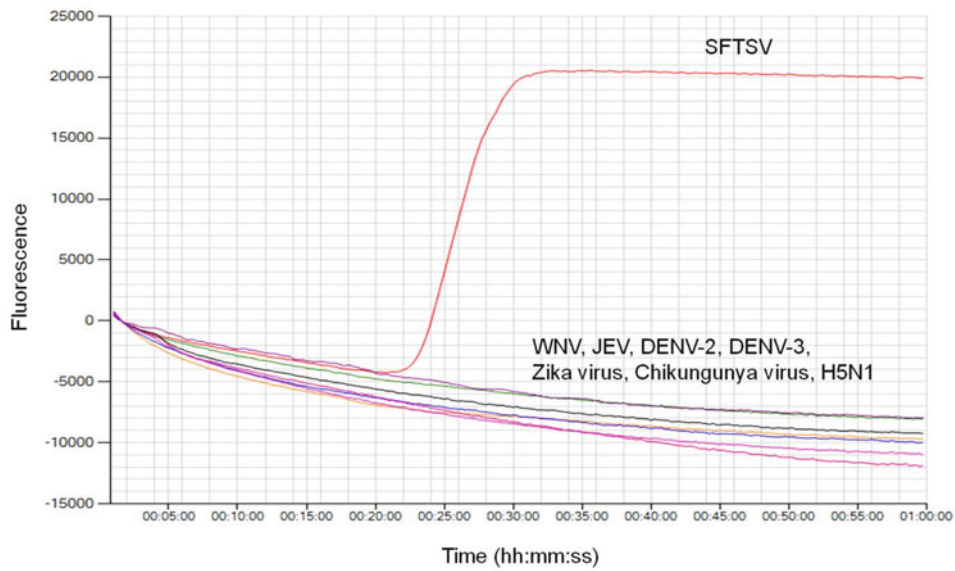
Fig. 1. Specificity of the real-time RT-LAMP assay for SFTSV. Except SFTSV, no other unspecific products were amplified by the real-time RT-LAMP assay. Control viruses: West Nile virus (WNV), Japanese encephalitis virus (JEV), dengue viruses (DENV-2 and DENV-3), Zika virus, chikungunya virus, and influenza virus (H5N1).
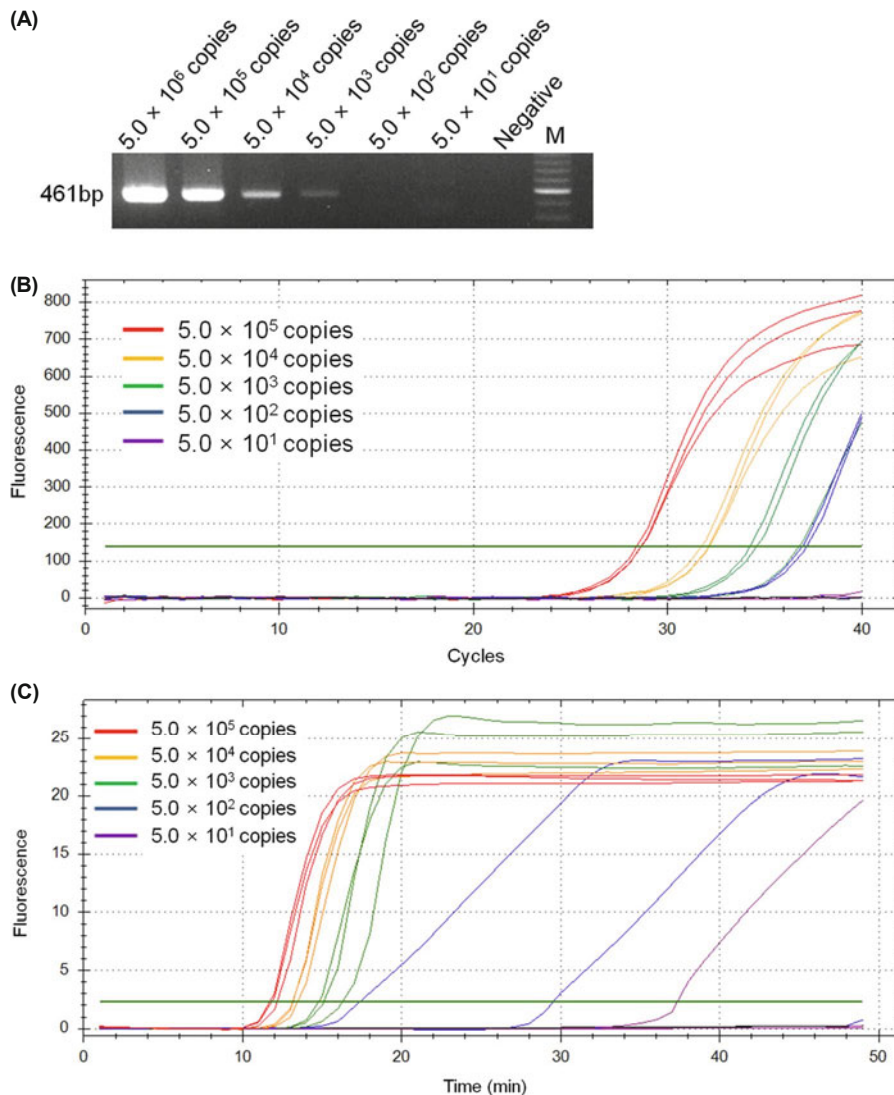

Fig. 2. SFTSV detection efficiency with different assays. SFTSV detection efficiencies were tested using dilutions of SFTSV RNA transcripts ranging from copy numbers of 5×10^6 to 5×10^1. (A) Agarose gels of conventional RT-PCR. The detection limit of the assay was 5×10^3 copies of RNA transcripts. M, molecular weight marker; Negative, negative control. (B) Real-time RT-PCR assay for SFTSV RNA transcripts. The detection limit was 5×10^2 copies of RNA transcripts. (C) Real-time RT-LAMP assay for SFTSV RNA transcripts. The detection limit was 5×10^2 copies of RNA transcripts.

positivity for SFTSV, while WNV, JEV, DENV-2, DENV-3, Zika virus, chikungunya virus, or H5N1 were not amplified (Fig. 1). Thus, real-time RT-LAMP can detect SFTSV with high specificity.

SFTSV detection efficiency

The SFTSV detection efficiencies of conventional PCR, real-time RT-PCR, and real-time RT-LAMP assays were determined using dilutions of SFTSV RNA transcripts ranging from 5×10^6 copies to 5×10^1 copies. The results confirmed that all PCR assays detected at least 5×10^2 copies to 5×10^3 copies of SFTSV RNA genes (Fig. 2). However, the efficiency of real-time RT-LAMP was higher because it was faster than the other two PCR assays. Real-time RT-LAMP determined positive results in 11 to 16 min compared with real-time RT-PCR, which determined positive results in 28 to 36 cycles with the same SFTSV RNA transcript copy number. Conventional RT-PCR takes at least 2 h to yield results on agarose gels, while real-time RT-PCR takes at least 30–40 min to obtain results. However real-time RT-LAMP can show the result in as little as 10–15 min with the same template conditions.

Discussion

SFTS is caused by SFTSV, which is a novel *Banyangvirus* of the *Phenuiviridae* family. Since 2009, more than 3,500 SFTS cases have been reported in China, Korea, and Japan (Li, 2015). The mortality rate for SFTS in South Korea was 47% in 2013 and 27% in 2015 (Park *et al.*, 2016). More efficient diagnostic methods are required to further reduce this mortality rate. The conventionally used methods for diagnosis are ELISA and quantitative real-time RT-PCR (Jiao *et al.*, 2012; Sun *et al.*, 2012). However, these methods have limitations such as being time-consuming and having low accuracy (Baek *et al.*, 2018). PCR-based molecular biological techniques have been widely adopted in diagnosis, and among them, the LAMP method in particular has extremely high sensitivity and specificity (Zeng *et al.*, 2018; Deng *et al.*, 2019). The real-time RT-LAMP method is rapid, highly specific, sensitive, and efficient, and can yield results in real time. Recently, the RT-LAMP assay has been utilized for the diagnosis of RNA viral pathogens, such as a novel swine acute diarrhea syndrome coronavirus and Japanese yam mosaic virus (Notomi *et al.*, 2000; Fukuta *et al.*, 2003; Wang *et al.*, 2018).

In this study, novel primers targeting the highly conserved L gene were designed for a real-time RT-LAMP assay (Table 1). The real-time RT-LAMP assay with these primers showed very high accuracy and specificity for SFTSV, without the amplification of the other control viruses (Fig. 1). Moreover, the real-time RT-LAMP assay showed more efficient results compared with conventional RT-PCR and real-time RT-PCR. The efficiency of the real-time RT-LAMP assay was over two-fold higher, from a copy number of 5×10^5 to 5×10^3, compared with real-time RT-PCR (Fig 2). Thus, real-time RT-LAMP is a more rapid and efficient method than real-time RT-PCR. Previous research has shown slightly more sensitive results using conventional one-step RT-PCR and quantitative one-step RT-PCR methods. However these methods take more than 1.5 h and then need an additional step for confirmation. Our novel primers and LAMP method can confirm SFTSV in 20 min in real time (Yoshikawa *et al.*, 2014).

SFTS is an endemic disease with a high mortality rate in Asian countries, especially South Korea (Choi *et al.*, 2016), warranting a novel primer set for real-time RT-LAMP to detect SFTSV isolates found in South Korea for early diagnosis and thus, a reduced mortality rate. Research groups in China and Korea have developed assays for SFTSV detection using real-time RT-LAMP with primers they designed to target S and L segments (Yang *et al.*, 2012; Huang *et al.*, 2014; Baek *et al.*, 2018). However, we designed a novel primer set targeting a totally different highly conserved region of the L segment, for detecting SFTSV isolated in South Korea using LAMP. Our laboratory-based method is accurate and provides objective results that can be viewed in real time, enabling a simpler and more rapid and sensitive molecular diagnosis.

Acknowledgments

Conflict of Interest

The authors declare no conflict of interest.

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