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Developing a multiplex loop-mediated isothermal amplification assay (LAMP) to determine severe fever with thrombocytopenia syndrome (SFTS) and scrub typhus

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Abstract

Severe fever with thrombocytopenia syndrome (SFTS) and scrub typhus are endemic zoonotic diseases that pose significant public health threats in East Asia. As these two diseases share common clinical features, as well as overlapping disease regions, it is difficult to differentiate between SFTS and scrub typhus. A multiplex reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed to detect large segments and GroES genes for SFTS virus (SFTSV) and *Orientia tsutsugamushi* (OT). The performance of the RT-LAMP assay was compared and evaluated with those of commercial Power-Chek™ SFTSV real-time PCR and LiliF™ TSUTSU nested PCR for 23 SFTS and 12 scrub typhus clinical samples, respectively. The multiplex SFTSV/OT/Internal control (IC) RT-LAMP assay showed comparable sensitivity (91.3%) with that of commercial PowerChek™ SFTSV Real-time PCR (95.6%) and higher sensitivity (91.6%) than that of LiliF™ TSUTSU nested PCR (75%). In addition, the multiplex SFTSV/OT RT-LAMP assay showed 100% specificity and no cross-reactivity for blood from uninfected healthy patients and samples from patients infected with other fever viruses. Thus, the multiplex SFTSV/OT/IC RT-LAMP assay could serve as a useful point-of-care molecular diagnostic test for SFTS and scrub typhus.

Introduction

Severe fever with thrombocytopenia syndrome (SFTS) and scrub typhus are endemic zoonotic diseases that are becoming significant public health threats in East Asia [1, 2]. The causative pathogen of SFTS is severe fever thrombocytopenia syndrome virus (SFTSV), which is a newly

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identified pathogenic member of the Phlebovirus species in the family Bunyaviridae [3]. The
SFTSV is usually transmitted by ticks such *as Haemophysalis longicornis* during outdoor activities [4]. SFTS has been mainly reported in East Asian countries, including Korea, China, and
Japan, with a high mortality rate (6.3–30%) [5–7]. The clinical features of SFTS are fever, headache, myalgia, and gastrointestinal symptoms, followed by thrombocytopenia and leukopenia
[8].

Scrub typhus is caused by *Orientia tsutsugamushi* (OT), an obligate intracellular bacterium
mediated by chigger mites such as *Leptotrombidium* species [9]. The distribution of scrub
typhus is thought to be endemic to the region known as the "tsutsugamushi triangle," which
includes China, Japan, Indonesia, Malaysia, Thailand, Pakistan, Korea, northern Australia,
and the islands of the western Pacific and Indian Ocean [10–12]. The mortality rate of scrub
typhus is 1.4% [13], and the clinical course of scrub typhus is usually mild and self-limiting;
however, delaying the treatment in severe cases can lead to complications such as renal failure,
myocarditis, meningoencephalitis, and death [14–16]. Unfortunately, scrub typhus is characterized by symptoms such as fever, headache, myalgia, cough, and abdominal pain, which are
difficult to differentiate from the symptoms of SFTS [8, 17]. Therefore, an incorrect diagnosis
of patients with these symptoms followed by inadequate treatment can lead to death [18].
Therefore, it is necessary to differentiate and diagnose SFTS and Scrub typhus in the early
stages [19].

Currently, the definitive diagnosis of scrub typhus and SFTS depends on laboratory-based
diagnostic methods, including indirect immunofluorescent antibody test (IFA), enzyme-
linked immunosorbent assay, immunochromatographic tests, and polymerase chain reaction
(PCR) [20–23]. The current diagnostic criterion for scrub typhus is IFA testing [24]. However,
single IFA measurements are sometimes insufficient for definitive diagnosis [12]. Recently,
PCR has been developed and used for the early diagnosis of scrub typhus [25, 26]. For the diagnosis of SFTS, RT-PCR is mainly used but not standardized; therefore, it is difficult to diagnose
SFTS quickly [27, 28]. In addition, since PCR tests are difficult to perform at regional hospitals
due to lack of adequate resources, samples obtained from patients suspected of SFTS are
mostly sent to the national laboratory, resulting in significant delays in PCR test results.
Recently, a simple version of a practical molecular assay, the loop-mediated isothermal amplification (LAMP) assay was developed for use in resource-poor environments [29, 30]. This
LAMP assay can be performed using simple equipment at a constant temperature, making it a
fast and cost-effective point-of-care diagnostic test [31, 32]. In addition, inexpensive multi-
channel isothermal amplifiers, such as Genie III (OptiGene, Horsham, UK) and T8-ISO
(TwistDX, Cambridge, UK), have been developed and commercialized.

In this study, we developed a multiplex SFTSV/OT/IC loop-mediated isothermal amplification (LAMP) assay for the differential diagnosis of SFTSV and *O. tsutsugamushi* infection,
based on a slightly modified DARQ probe method [33, 34]. The detection limit of the multiplex SFTSV/OT/IC RT-LAMP assay was confirmed using diluted SFTS and scrub typhus clinical samples. The sensitivity and specificity of the RT-LAMP assay were compared and
evaluated with those of the commercial PowerChek™ SFTSV Real-time PCR kit and LiliF™
TSUTSU nested PCR kit for SFTS and scrub typhus clinical samples, respectively.

Materials and methods

Clinical samples and nucleic acid extraction

We collected 35 clinical samples of serum and blood from 19 patients suspected of being
infected with SFTSV (23 from 7 patients) and *O. tsutsugamushi* (12 from 12 patients) in the
Republic of Korea. SFTS blood/serum samples (23) were collected on different days from

hospitalized 7 SFTS patients. SFTS (23) and scrub typhus (12) clinical samples were confirmed using the SFTSV qRT-PCR and *O. tsutsugamushi* qPCR, which were previously reported by Yoshikawa *et. al.* [35] and Tantibhedhyangkul *et. al.* [26], respectively. To assess the specificity of the multiplex SFTSV/OT/IC RT-LAMP assay, 100 clinical sample specimens from individuals with (38) and without (62) other viral infections were tested. Viral infection samples, as confirmed via PCR using qRT-PCR [36–38] and the Anyplex™ II RV16 detection kit (Seegene, Seoul, South Korea), included 1 Hantaan virus, 4 Dengue virus (1–4), 1 Chikungunya virus, 4 influenza virus A/H1N1, 4 influenza virus A/H3N2, 4 influenza virus B, 4 respiratory syncytial virus (RSV) A, 4 RSV B, and 12 coronaviruses (KHU1, NL63, 229E). Nucleic acids (DNA and RNA), were extracted from 200 μL of clinical blood or serum samples using the cell DNA/RNA/NA kit (Genolution, Seoul, Korea) with Nextractor® NX-48S (Genolution, Seoul, Korea), according to the manufacturer's instructions. Nucleic acid samples were stored at -50 °C before further testing. This study was approved by the Medical Ethics Committee of Korea University Guro Hospital (IRB No. 2020GR0556). Informed consent was waived by the institutional review board (IRB) because this study used residual samples.

Primer design

The RT-LAMP primer sets for SFTSV and *O. tsutsugamushi* were designed from the conserved regions of the L segment and groES genes (Table 1). The actin beta gene in humans was used as an internal control (IC), as previously reported [39]. All LAMP primers including two outer primers (forward primer F3 and backward primer B3), two inner primers (forward inner primer FIP and backward inner primer BIP), and two loop primers (forward loop primer LF and backward loop primer LB) were designed using the Primer Explorer v4 software (Eiken Chemical Co., Tokyo, Japan). For multiple LAMPs, we used a slightly modified DARQ probe method. Briefly, a dye-labeled artificial nucleic acid + FLP sequence probe and a quencher-labeled displacement probe complementary to the artificial nucleic acid sequence were used. In this study, two types of artificial nucleic acids (32mers and 35mers) were used for multiplexing different fluorescence (FAM/Hex and Cy5) quenched by BHQ1 and BHQ2, respectively. A FAM (or Hex)-labeled 32-artificial oligomer-SFTS (or IC) FLP was designed for SFTS (or IC) FLP probe 1 and a Cy5-labeled 35-artificial oligomer-OT FLP was designed for tsu FLP probe 2. The quencher-labeled 30-oligonucleotide (BHQ1) or 35-oligonucleotide (BHQ2) was complementary to artificial nucleic acids sequences of SFTS (or IC) FLP probe 1 and tsu FLP probe 2, respectively. Before use in LAMP, all primers were assessed for specificity by performing a BLAST search. All LAMP primers and probes were synthesized by Macrogen (Seoul, South Korea).

Real-time RT-PCR

To confirm SFTS and scrub typhus positive clinical samples, the SFTSV L, M, and S gene qRT-PCR primer sets [35] and *O. tsutsugamushi* groEL gene qPCR primer set [26] were used, and PCR conditions were set according to the protocol described by Yoshikawa *et al.*, and Tantibhedhyangkul *et al.*, respectively. The thermocycling parameters of the SFTSV L segment gene qRT-PCR were used as follows: after incubation at 95˚C for 2 min, a reverse transcription step was performed at 55˚C for 30 min, followed by 45 cycles of 94˚C for 30 s, 52˚C for 30 s with fluorescence detection, and 68˚C for 30 s. The PCR cycling conditions for *O. tsutsugamushi* groEL gene qPCR were as follows: initial denaturation and hot-start enzyme activation at 95˚C for 3 min, followed by 45 cycles of denaturation at 95˚C for 1 s, combined annealing/extension at 58˚C for 30 s with data acquisition for 12 s, and extension at 70˚C for 3 s.

Table 1. The multiplex SFTSV/OT/IC RT-LAMP primer sets.

Target	Name	Sequence (5´-3´)	Length (mer)	Conc of LAMP primer mix (20x)
SFTSV (L segment gene)	SFTSV F3	CCG ACT CAG GCT TTG GTT C	19	4 μM
	SFTSV B3	AAG GCA GGC TTG AAT CGC	18	4 μM
	SFTSV FIP	CTA CCC CCT CCA CAT TGC CYT TRA TGG ATC ATG GCG CAT A	40	32 μM
	SFTSV BIP	TCA GTG ACC CTG CAA AAG ARC TTT TGC CTC TTT GGG GGT RTC	42	32 μM
	SFTSV FLP	TTG CCC ACA GCT CAC CA	17	4 μM
	SFTSV BLP	CAT TGC YAT CTC TGA TGA TCC AGA W	25	10 μM
	SFTSV FLP probe1	FAM-CGG GCC CGT ACA AAG GGA ACA CCC ACA CTC CGT TGC CCA CAG CTC ACC A	49	6 μM
O. tsutsugamushi (GroES gene)	tsu F3	GAT TAT ATG AAA TAC CAA CCA CTG	24	4 μM
	tsu B3	TTT CAG TAC CAG CCC ATT	18	4 μM
	tsu FIP	CCT TTG CGG TAT CTG GAA TAA GAA TTA TGA TCG TGT GCT AGT TGA G	46	32 μM
	tsu BIP	CAG AGG GAA TAG TAG TTA TGG TTG GCC TTT CTT TAC TTT TAA CGG TGT A	49	32 μM
	tsu FLP	TTT ACC RTG TGC TTC ATC ATT GTG	24	4 μM
	tsu BLP	CGG GGG CTA TAG AAA TGA TAA AGG	24	10 μM
	tsu FLP probe2	CY5-GTC AGT GCA GGC TCC CGT GTT AGG ACG AGG GTA GGC GGG GGC TAT AGA AAT GAT AAA GG	59	6 μM
Internal control (Actin beta)	ACTB F3	AGT ACC CCA TCG AGC ACG	18	4 μM
	ACTB B3	AGC CTG GAT AGC AAC GTA CA	20	4 μM
	ACTB FIP	GAG CCA CAC GCA GCT CAT TGT ATC ACC AAC TGG GAC GAC A	40	32 μM
	ACTB BIP	CTG AAC CCC AAG GCC AAC CGG CTG GGG TGT TGA AGG TC	38	32 μM
	ACTB BLP	TGT GGT GCC AGA TTT TCT CCA	21	4 μM
	ACTB FLP	CGA GAA GAT GAC CCA GAT CAT GT	23	10 μM
	ACTB FLP probe1	HEX-CGG GCC CGT ACA AAG GGA ACA CCC ACA CTC CGC GAG AAG ATG ACC CAG ATC ATG T	55	6 μM
Quencher probe 1		GAG TGT GGG TGT TCC CTT TGT ACG GGC CCG-BHQ1	30	9 μM
Quencher probe 2		CCT ACC CTC GTC CTA ACA CGG GAG CCT GCA CTG AC-BHQ2	35	9 μM

PowerChek™ SFTSV Real-time PCR and LiliF™ TSUTSU nested PCR were carried out according to a manufacture's protocol.

Multiplex RT-LAMP

For multiplex SFTSV/OT/IC RT-LAMP assay, the reaction mixture was prepared with 12.5 μL of 2× reaction buffer, 1.25 μL of SFTSV LAMP primer mix (20x), 1.5 μL of OT LAMP primer mix (20x), 0.625 μL of IC (actin β) LAMP primer mix (20x), 1.875 μL of 9 μM quencher 1 solution for quenching the SFTS and IC LAMP probe, 1.5 μL of 9 μM quencher 2 solution for quenching the OT LAMP probe, and 5 μL of sample RNA (with a final reaction volume of 25 μL). The compositions of all LAMP primer mix (20x) were 4 μM of two outer primers (F3 and B3) and 32 μM of two inner primers (FIP and BIP), 10 μM of loop BLP primer, 4 μM loop FLP primer, and 6 μM loop FLP probe primer. The RT-LAMP assay was run on a CFX 96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) at 62ºC for 40 min. The FAM, Hex, and Cy5 fluorescence channels were used to detect SFTSV, IC, and *O. tsutsugamushi*, respectively. For comparative studies, the performance of multiplex SFTSV/OT/IC LAMP assays was compared with that of commercial PowerChek™ SFTSV Real-time PCR kit and LiliF™ TSUTSU nested PCR Kit. The RT-LAMP assay was performed using the RT-LAMP 2× Master Mix (ELPIS-Biotech, Daejeon, South Korea).

Limits of detection

pTOP Blunt V2 plasmids, including partial L segment gene sequences of SFTSV and groES gene sequences of *O. tsutsugamushi*, were used to test the limit of detection (LOD) of the RT-LAMP assay. All plasmids were constructed by Macrogen. To determine the LOD of the monoplex SFTSV RT-LAMP, monoplex OT RT-LAMP, and multiplex SFTSV/OT/IC RT-LAMP assays, the plasmids were serially diluted 10-fold from 1×10^6 to 1×10^0 copies/μL. This test was repeated 3 times. In addition, the LODs of the monoplex SFTSV RT-LAMP, monoplex OT RT-LAMP, and multiplex SFTSV/OT/IC RT-LAMP assays were compared with those of the commercial PowerChek™ SFTSV Real-time PCR kit and LiliF™ TSUTSU nested PCR kit for 2-fold serial dilutions of clinical samples from patients infected with SFTSV or *O. tsutsugamushi*. According to FDA EUA guidelines for COVID-19 diagnostic tests, the LODs of the monoplex SFTSV RT-LAMP, monoplex OT RT-LAMP, and multiplex SFTSV/OT/IC RT-LAMP assays were determined as the minimum concentration in a 2-fold dilution series at which 19 of 20 replicates amplify [40, 41]. the LODs of commercial PowerChek™ SFTSV Real-time PCR kit and LiliF™ TSUTSU nested PCR kit were repeated five times and determined as the minimum concentration in a 2-fold dilution series at which 5 of 5 replicates amplify.

Results

Optimization of multiplex SFTSV/OT/IC RT-LAMP assay

The sensitivities of the monoplex SFTSV (FAM) and monoplex OT (Hex) RT-LAMP assay using strand-displaceable probes were evaluated by testing synthetic plasmid standards, including synthetic partial L segment gene of SFTSV and groES genes of *O. tsutsugamushi* ranging from 10^6 to 10^0 copies/μL, respectively. The limits of detection of monoplex SFTSV RT-LAMP and monoplex OT RT-LAMP were $1x10^2$ copies/μL and $1x10^1$ copies/μL, respectively (Table 2). For optimization of multiplex SFTSV /OT (Cy5)/IC (Hex) RT-LAMP assays, different ratios (1.5:2:0.6, 1.5:1.5:0.6 and 2:1.5:0.6) of LAMP primers set (vol, μL/ 25 μL reaction) were tested at 62ºC with synthetic plasmids including partial L segment gene of SFTSV and groES genes of *O. tsutsugamushi* (Fig 1A). For internal control (IC), actin beta LAMP primer set was used for the human actin beta gene amplification (Table 1). In SFTSV signal detection, a ratio of 1.5:1.5:0.6 showed faster Ct values (8.27) than others (1.5:2:0.6 = 8.94 and 2:1.5:0.6 = 8.30). In OT signal detection, a ratio of 1.5:2:0.6 showed faster Ct values (7.84) than others (1.5:1.5:0.6 = 9.18 and 2:1.5:0.6 = 9.59). Comprehensively considering the ct values and RFUs, the 1.5:2:0.6 ratio of the SFTS/OT/IC LAMP primer set was finally determined. Next, temperature-gradient tests (59, 62 and 65ºC) showed that the optimum temperature was 62ºC for multiplex SFTSV/OT/IC RT-LAMP assay (Fig 1B). Finally, in LOD test, the multiplex SFTSV/OT/IC RT-LAMP assay showed the same detection limit of monoplex SFTSV and OT RT-LAMP primer set for synthetic SFTSV and OT plasmid standards (Table 2).

Comparison of detection limits of the multiplex SFTSV/OT/IC RT-LAMP assay with those of commercial kits for serial diluted SFTS and scrub typhus clinical samples

The LODs of the monoplex and multiplex SFTSV/OC/IC LAMP primer sets were compared to those of two commercial kits, the PowerChek™ SFTSV Real-time PCR kit (Kogene Biotech, Seoul, Korea) and LiliF™ TSUTSU nested PCR kit (iNtRON, Seongnam-Si, South Korea), for 2-fold serial diluted SFTSV serum samples and scrub typhus blood samples (range 2^8–2^{15} and 2^1–2^8, respectively) (Table 3). For serial two-fold diluted SFTS clinical samples, monoplex and PowerChekTM SFTSV Real-time PCR kit showed the same LODs of 2^{10}, whereas the LOD of

Table 2. Limit of detection test for the monoplex and multiplex SFTSV/OT/IC LAMP primer set.

	Plasmid	Monoplex RT-LAMP		Multiplex SFTSV/OT/IC RT-LAMP					
		SFTSV		SFTSV		IC		O. tsutsugamushi	
		Ct	RFU	Ct	RFU	Ct	RFU	Ct	RFU
SFTSV plasmid dilution sample (copies/ µL)	10^6	7.6	17581	8.5	21559	N/A	71.7	N/A	-39.1
	10^5	9.3	17842	10.5	20796	N/A	75.8	N/A	241
	10^4	10.1	17638	11.6	20939	N/A	73.3	N/A	290
	10^3	13.3	17760	12.7	21164	N/A	66	N/A	318
	10^2	17.5	18137	20.8	21961	N/A	45.1	N/A	297
	10^1	N/A	187	N/A	7	N/A	-0.348	N/A	7.55
	10^0	N/A	56	N/A	49	N/A	3.61	N/A	-1.77
	DW	N/A	134	N/A	97	N/A	3.45	N/A	15.5
	Plasmid	O. tsutsugamushi		SFTSV		IC		O. tsutsugamushi	
		Ct	RFU	Ct	RFU	Ct	RFU	Ct	RFU
***O. tsutsugamushi* plasmid dilution sample (copies/ µL)**	10^6	6.8	3939	N/A	-8.32	N/A	-4.81	8.1	5240
	10^5	7.9	4228	N/A	-8.11	N/A	126	9.4	5995
	10^4	9.7	4420	N/A	-7.79	N/A	-7.44	11.9	6079
	10^3	10.8	4351	N/A	-1.47	N/A	-6.33	11.8	6045
	10^2	11.9	4371	N/A	-2.59	N/A	-6	12.9	5782
	10^1	15	4081	N/A	-10.1	N/A	-5.53	15.2	5992
	10^0	18.4	4184	N/A	0.334	N/A	2.33	21.4	5921
	DW	N/A	42	N/A	23.1	N/A	3.96	N/A	25

Each mean value of Ct and RFU is the average of the three LAMP assay repetitions.
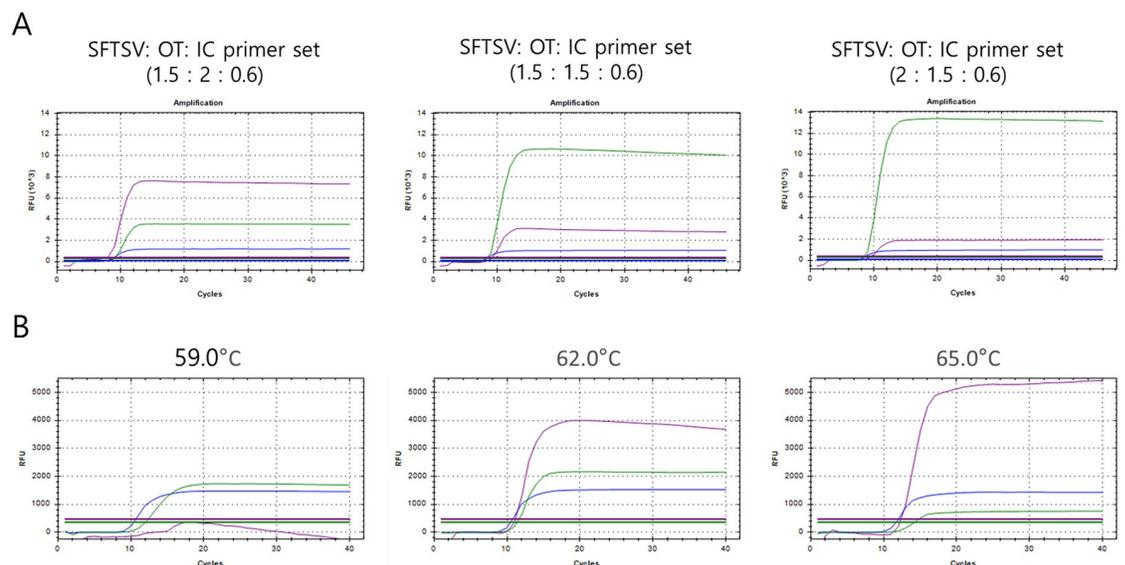
Fig 1. Optimization of the multiplex SFTSV/OT/IC LAMP primer set. (A) Different concentration ratios of SFTSV, OT, and IC primer sets (1.5:2:0.6, 1.5:1.5:0.6, and 2:1.5:0.6, respectively) for SFTSV, OT, and IC plasmid mixtures (1:1:1). (B) Temperature gradient tests (59–65˚C) of the multiplex SFTSV/OT/IC LAMP assay.

Table 3. Limit of detection tests of the multiplex SFTSV/OT/IC RT-LAMP assay, PowerChek™ SFTSV Real-time PCR kit and LiliF™ TSUTSU nested PCR kit for two-fold diluted clinical samples from patients infected with SFTSV or *O. tsutsugamushi*.

Assays	Clinical samples (Nucleic acid extracted from clinical sample (2^n) per reaction)															
	SFTSV								*O. tsutsugamushi*							
	8	9	10	11	12	13	14	15	1	2	3	4	5	6	7	8
Monoplex SFTSV RT-LAMP	▨	▨	▨	▨	▨											
Monoplex OT RT-LAMP									▨	▨	▨	▨	▨			
Multiplex SFTSV/OT/IC RT-LAMP	▨	▨	▨	▨					▨	▨	▨	▨	▨			
PowerChek™ SFTSV Real-time PCR kit	▨	▨	▨	▨	▨											
LiliF™ TSUTSU nested PCR kit									▨	▨	▨					

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multiplex SFTSV/OC/IC LAMP assay was 2^{12}, which is one step lower than results of others. However, for scrub typhus clinical samples, both of the monoplex and multiplex SFTSV/OC/IC LAMP assay showed the superior LOD (2^5) to that (2^3) of the LiliFTM TSUTSU nested PCR kit.

Comparison of the clinical performance of the multiplex SFTSV/OT/IC RT-LAMP assay with those of the commercial PowerChek™ SFTSV Real-time PCR kit and LiliF™ TSUTSU nested PCR kit using clinical samples

To confirm the clinical performance of the multiplex SFTSV/OT/IC RT-LAMP assay, the sensitivities of the assays were compared to those of the commercial PowerChek™ SFTSV Real-time PCR kit and LiliF™ TSUTSU nested PCR kit for 35 clinical samples from patients infected with SFTSV (23) and *O. tsutsugamushi* (12). For the specificities of these three assays, 100 clinical sample specimens from individuals with (38) and without (62) other viral infections were used (Table 4). For the SFTS clinical samples (n = 23), the sensitivity of the PowerChek™ SFTSV Real-time PCR kit was 95.6% (S gene: 95.6%, and M genes: 86.9%). The sensitivity of the multiplex SFTSV/OT/IC RT-LAMP assay was 91.3%. For scrub typhus clinical samples (n = 12), the sensitivity of the LiliF™ TSUTSU nested PCR kit was 75% and that of the multiplex SFTSV/OT/IC RT-LAMP assay was 91.6%. Overall, the multiplex SFTSV/OT/IC RT-LAMP assay showed similar sensitivities to that of the commercial PowerChek™ SFTSV Real-time PCR kit and superior sensitivity to the LiliF™ TSUTSU nested PCR kit. For 100

Table 4. Comparison of clinical performance between the multiplex SFTSV/OT/IC RT-LAMP assay, PowerChek™ SFTSV Real-time PCR kit, and LiliF™ TSUTSU nested PCR kit for clinical samples from patients infected with SFTSV or *O. tsutsugamushi*.

Clinical samples		Multiplex SFTSV/OT/IC RT-LAMP			PowerChek™ SFTSV Real-time PCR kit				LiliF™ TSUTSU nested PCR kit
		SFTSV (FAM)	IC (Hex)	OT (Cy5)	M gene (FAM)	S gene (Hex)	M+S gene	IC (Cy5)	
SFTSV (n = 23)	P/N	21/2	22/1	0/23	20/3	22/1	22/1	23/0	-
	Sensitivity	**91.3%**	**95.6%**	-	**86.9%**	**95.6%**	**95.6%**	**100%**	-
	Specificity	-	-	**100%**	-	-	-	-	-
O. tsutsugamushi (n = 12)	P/N	0/12	8/4	11/1	-	-	-	-	9/3
	Sensitivity	-	**66.7%**	**91.6%**	-	-	-	-	**75%**
	Specificity	100%	-	-	-	-	-	-	-
Non-infection (n = 100)	P/N	0/100	94/6	0/100	0/100	0/100	0/100	98/2	2/98
	Sensitivity	-	**94%**	-	-	-	-	**98%**	-
	Specificity	**100%**	-	**100%**	**100%**	**100%**	**100%**	-	**98%**

The sensitivities and specificities were calculated by taking the results of reference SFTSV qRT-PCR and tsutsugamushi qPCR as a standard. P/N: positive/negative ratio

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negative clinical samples (non-infection/other viral infections; n = 64/38), the specificity of the two assays was 100%, except for the LiliF™ TSUTSU nested PCR kit (98%) (Table 4).

Cross-reactivity tests of SFTSV/OT/IC RT-LAMP assay with other fever viruses

To confirm the absence of cross-reactivity with other common fever viruses, a total of 38 viral RNA samples, including Hantaan virus, Dengue virus, chikungunya virus, influenza virus A, influenza virus B, RSV A, RSV B, coronavirus 229E, NL63, and OC43, were tested using the multiplex SFTSV/OT/IC RT-LAMP assay (Table 5). As a result, the multiplex SFTSV/OT/IC RT-LAMP assay did not show cross-reactivity for all the tested samples.

Discussion

Severe fever with thrombocytopenia syndrome virus (SFTSV) and *O. tsutsugamushi* are not transmitted by the same vectors; however, these two diseases share a common point as they are transmitted by arthropod bites mostly during outdoor activities [18, 42]. In addition, these two diseases share common clinical features, such as fever, nausea, vomiting, diarrhea, headache, and muscle pain, as well as overlapping disease regions. Furthermore, many cases of co-infection with SFTSV and *O. tsutsugamushi* have been reported [43–45].

Here, we developed a multiplex SFTSV/OT/IC RT-LAMP assay to detect SFTSV L segment, groES, and actin beta genes. In sensitivity tests for SFTS and scrub typhus clinical samples, the multiplex SFTSV/OT/IC RT-LAMP assay showed 91.3% and 91.6% for SFTS (n = 23) and scrub typhus (n = 12) clinical samples, respectively. These results are comparable to those (M gene: 86.9% and S gene: 95.6%) of the commercial PowerChek™ SFTSV Real-time PCR, and superior to 75% of LiliF™ TSUTSU nested PCR kit. The sensitivity and specificity results of PowerChek™ SFTSV Real-time PCR were within the scope of previous results (sensitivity: 100% (CI: 73.2%–100%) and sensitivity: 98.1% (CI: 92.5%–99.7%)) reported by Yoo. et al [46]. Therefore, the results of this test are considered reliable, although they were tested with a small number of samples. In addition, the LODs for the multiplex SFTSV/OT/IC RT-LAMP for

Table 5. Cross-reactivity of the multiplex SFTSV/*OT*/IC RT-LAMP assay against other fever infectious viruses.

Virus	No	Multiplex SFTSV/OT/IC RT-LAMP		
		SFTSV (FAM)	IC (Hex)	OT (Cy5)
HANV	1	0/1	0/1	0/1
DENV 1–4	4	0/4	3/4	0/4
CHIKV	1	0/1	0/1	0/1
Inf A H1	4	0/4	4/4	0/4
Inf A H3	4	0/4	4/4	0/4
Inf B	4	0/4	4/4	0/4
229E	4	0/4	4/4	0/4
NL63	4	0/4	4/4	0/4
OC43	4	0/4	4/4	0/4
RSV A	4	0/4	4/4	0/4
RSV B	4	0/4	4/4	0/4

HANV: hantaan virus; DENV: dengue virus; CHIKV: chikungunya virus; Inf A H1: Influenza A H1; Inf A H3: Influenza A H3; Inf B: Influenza B; 229E: human coronavirus 229E; NL63: human coronavirus NL63; OC43: human coronavirus OC43; RSV A: respiratory syncytial virus A; RSV B: respiratory syncytial virus B.

diluted SFTS and scrub typhus clinical samples were the same as those of the PowerChek™ SFTSV Real-time PCR and LiliF™ TSUTSU nested PCR kit, respectively.

To simultaneously detect SFTSV and *O. tsutsugamushi*, nucleic acid (DNA/RNA) extraction from whole blood is an important step in multiplex SFTSV/OT/IC RT-LAMP assay. Here, Nextractor® NX-48S, which is an automated nucleic acid extraction instrument, was used for fast (within 20 min) and contamination-free extraction of nucleic acids from serum or whole blood. Unfortunately, the multiplex SFTSV/OT/IC RT-LAMP assay could not detect OT signals with nucleic acids (DNA/RNA) extracted from the serum of patients infected with *O. tsutsugamushi*. This may be because *O. tsutsugamushi* is present at low concentrations in the serum. In fact, most of the *O. tsutsugamushi* qPCR and LAMP assays developed to date were performed using DNA extracted from whole blood, buffy coat, and eschars of patients infected with *O. tsutsugamushi* [30, 47, 48]. In the sensitivity test, we used 24 SFTS serum clinical samples to confirm the performance of the multiplex SFTSV/OT/IC RT-LAMP assay because of the small number of SFTS whole blood samples. Thus, to confirm whether the multiplex SFTSV/OT/IC RT-LAMP assay can detect SFTSV in whole blood samples, an additional 12 whole blood samples, which were matched with the positive SFTS serum sample, were tested and confirmed to be positive in the multiplex SFTSV/OT/IC RT-LAMP assay (S1 Table).

Our study has some limitations. First, the multiplex SFTSV/OT/IC RT-LAMP assay was performed with a relatively small sample size of positive SFTS (23) and scrub typhus (12) clinical samples, which resulted in widened confidence intervals for our estimates of diagnostic accuracy. However, considering that the multiplex SFTSV/OT/IC RT-LAMP assay showed the same or higher sensitivity compared to the two commercially available diagnostic kits, and 100% specificity to the negative samples, the multiplex SFTSV/OT/IC RT-LAMP assay is sufficiently competitive in commercial development. In addition, it can be applied quickly and cost-effectively, particularly in the early stages of fever in patients in endemic areas. Second, in SFTS LOD test, we used SFTS DNA plasmid as a standard material for LOD test of the multiplex SFTSV/OT/IC RT-LAMP assay. However, there is the deficiency of characterizing an RT-LAMP assay with a DNA standard because it cannot confirm the function of reverse transcriptase. Thus, using SFTS and scrub typhus clinical samples, we reconfirmed the limit of detection of monoplex and multiplex SFTSV/OT/IC RT-LAMP assay compared to those of commercial LiliF™ TSUTSU nested PCR and PowerChekTM SFTSV Real-time PCR kit.

In this study, we developed a multiplex SFTSV/OT/IC RT-LAMP assay capable of simultaneous diagnosis of SFTS and scrub typhus within 40 min. For sensitivity and specificity tests with SFTS and scrub typhus clinical samples, the multiplex SFTSV/OT/IC RT-LAMP assay showed similar or superior performance compared with commercial PowerChek™ SFTSV Real-time PCR and LiliF™ TSUTSU nested PCR. Thus, the multiplex SFTSV/OT/IC RT-LAMP assay could serve as a useful point-of-care molecular diagnostic test for SFTS and scrub typhus.

Supporting information

S1 Table. Sensitivities the multiplex SFTSV/OT/IC RT-LAMP assay for whole blood SFTS clinical samples.
(DOCX)

S1 File. Raw data.
(XLSX)

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References

1. Kirino Y, Ishijima K, Miura M, Nomachi T, Mazimpaka E, Sudaryatma PE, et al. Seroprevalence of severe fever with thrombocytopenia syndrome virus in small-animal veterinarians and nurses in the Japanese Prefecture with the highest case load. Viruses. 2021; 13: 229. Epub 2021/02/06. https://doi.org/10.3390/v13020229 PMID: 33540629.

2. Elliott I, Pearson I, Dahal P, Thomas NV, Roberts T, Newton PN. Scrub typhus ecology: a systematic review of Orientia in vectors and hosts. Parasites & vectors. 2019; 12: 513. https://doi.org/10.1186/s13071-019-3751-x PMID: 31685019

3. Yu XJ, Liang MF, Zhang SY, Liu Y, Li JD, Sun YL, et al. Fever with thrombocytopenia associated with a novel bunyavirus in China. N Engl J Med. 2011; 364: 1523–1532. https://doi.org/10.1056/NEJMoa1010095 PMID: 21410387

4. Chung JK, Kim CM, Kim DM, Yun NR, Park JW, Seo J, et al. Severe fever with thrombocytopenia syndrome associated with manual de-ticking of domestic dogs. Vector Borne Zoonotic Dis. 2020; 20: 285–294. https://doi.org/10.1089/vbz.2019.2463 PMID: 32045336

5. Ding F, Zhang W, Wang L, Hu W, Soares Magalhaes RJ, Sun H, et al. Epidemiologic features of severe fever with thrombocytopenia syndrome in China, 2011–2012. Clin Infect Dis. 2013; 56: 1682–1683. https://doi.org/10.1093/cid/cit100 PMID: 23429379

6. Yun SM, Park SJ, Kim YI, Park SW, Yu MA, Kwon HI, et al. Genetic and pathogenic diversity of severe fever with thrombocytopenia syndrome virus (SFTSV) in South Korea. JCI Insight. 2020; 5: e129531. https://doi.org/10.1172/jci.insight.129531 PMID: 31877113

7. Silvas JA, Aguilar PV. The emergence of severe fever with thrombocytopenia syndrome virus. Am J Trop Med Hyg. 2017; 97: 992–996. https://doi.org/10.4269/ajtmh.16-0967 PMID: 28820686

8. Choi SJ, Park SW, Bae IG, Kim SH, Ryu SY, Kim HA, et al. Severe fever with thrombocytopenia syndrome in South Korea, 2013–2015. PLoS Negl Trop Dis. 2016; 10: e0005264. https://doi.org/10.1371/journal.pntd.0005264 PMID: 28033338

9. Kim G, Ha NY, Min CK, Kim HI, Yen NT, Lee KH, et al. Diversification of Orientia tsutsugamushi genotypes by intragenic recombination and their potential expansion in endemic areas. PLoS Negl Trop Dis. 2017; 11: e0005408. https://doi.org/10.1371/journal.pntd.0005408 PMID: 28248956

10. Jiang J, Richards AL. Scrub typhus: no longer restricted to the tsutsugamushi triangle. Trop Med Infect Dis. 2018; 3: 11. https://doi.org/10.3390/tropicalmed3010011 PMID: 30274409

11. Janardhanan J, Trowbridge P, Varghese GM. Diagnosis of scrub typhus. Expert Rev Anti Infect Ther. 2014; 12: 1533–1540. https://doi.org/10.1586/14787210.2014.974559 PMID: 25359599

12. Blacksell SD, Bryant NJ, Paris DH, Doust JA, Sakoda Y, Day NP. Scrub typhus serologic testing with the indirect immunofluorescence method as a diagnostic gold standard: a lack of consensus leads to a lot of confusion. Clin Infect Dis. 2007; 44: 391–401. https://doi.org/10.1086/510585 PMID: 17205447

13. Bonell A, Lubell Y, Newton PN, Crump JA, Paris DH. Estimating the burden of scrub typhus: a systematic review. PLoS Negl Trop Dis. 2017; 11: e0005838. https://doi.org/10.1371/journal.pntd.0005838 PMID: 28945755

14. Kim DM, Kim SW, Choi SH, Yun NR. Clinical and laboratory findings associated with severe scrub typhus. BMC Infect Dis. 2010; 10: 108. https://doi.org/10.1186/1471-2334-10-108 PMID: 20433689

15. Chrispal A, Boorugu H, Gopinath KG, Prakash JA, Chandy S, Abraham OC, et al. Scrub typhus: an unrecognized threat in South India—clinical profile and predictors of mortality. Trop Doct. 2010; 40: 129–133. https://doi.org/10.1258/td.2010.090452 PMID: 20360426

16. Crecelius EM, Burnett MW. Scrub Typhus. J Spec Oper Med. 2020; 20: 120–122. Epub 2020/03/24. PMID: 32203616.

17. Rajapakse S, Weeratunga P, Sivayoganathan S, Fernando SD. Clinical manifestations of scrub typhus. Trans R Soc Trop Med Hyg. 2017; 111: 43–54. https://doi.org/10.1093/trstmh/trx017 PMID: 28449088

18. Park SW, Lee CS, Kim JH, Bae IG, Moon C, Kwak YG, et al. Severe fever with thrombocytopenia syndrome: comparison with scrub typhus and clinical diagnostic prediction. BMC Infect Dis. 2019; 19: 174. https://doi.org/10.1186/s12879-019-3773-1 PMID: 30782137

19. Yun JH, Hwang HJ, Jung J, Kim MJ, Chong YP, Lee SO, et al. Comparison of chest radiographic findings between severe fever with thrombocytopenia syndrome and scrub typhus: Single center observational cross-sectional study in South Korea. Medicine. 2019; 98: e17701. https://doi.org/10.1097/MD.0000000000017701 PMID: 31725613

20. Seo JW, Kim D, Yun N, Kim DM. Clinical update of severe fever with thrombocytopenia syndrome. Viruses. 2021; 13: 1213. https://doi.org/10.3390/v13071213 PMID: 34201811

21. Chakraborty S, Sarma N. Scrub typhus: an emerging threat. Indian J Dermatol. 2017; 62: 478–485. https://doi.org/10.4103/ijd.IJD_388_17 PMID: 28979009

22. Li DX. Severe fever with thrombocytopenia syndrome: a newly discovered emerging infectious disease. Clin Microbiol Infect. 2015; 21: 614–620. https://doi.org/10.1016/j.cmi.2015.03.001 PMID: 25769426

23. Kala D, Gupta S, Nagraik R, Verma V, Thakur A, Kaushal A. Diagnosis of scrub typhus: recent advancements and challenges. 3 Biotech. 2020; 10: 396. https://doi.org/10.1007/s13205-020-02389-w PMID: 32834918

24. Pradutkanchana J, Silpapojakul K, Paxton H, Pradutkanchana S, Kelly DJ, Strickman D. Comparative evaluation of four serodiagnostic tests for scrub typhus in Thailand. Trans R Soc Trop Med Hyg. 1997; 91: 425–428. https://doi.org/10.1016/s0035-9203(97)90266-2 PMID: 9373640

25. Kim DM, Park G, Kim HS, Lee JY, Neupane GP, Graves S, et al. Comparison of conventional, nested, and real-time quantitative PCR for diagnosis of scrub typhus. J Clin Microbiol. 2011; 49: 607–612. https://doi.org/10.1128/JCM.01216-09 PMID: 21068287

26. Tantibhedhyangkul W, Wongsawat E, Silpasakorn S, Waywa D, Saenyasiri N, Suesuay J, et al. Use of multiplex real-time PCR to diagnose scrub typhus. J Clin Microbiol. 2017; 55: 1377–1387. https://doi.org/10.1128/JCM.02181-16 PMID: 28202789

27. Sun Y, Liang M, Qu J, Jin C, Zhang Q, Li J, et al. Early diagnosis of novel SFTS bunyavirus infection by quantitative real-time RT-PCR assay. J Clin Virol. 2012; 53: 48–53. https://doi.org/10.1016/j.jcv.2011.09.031 PMID: 22024488

28. Augustine R, Hasan A, Das S, Ahmed R, Mori Y, Notomi T, et al. Loop-mediated isothermal amplification (LAMP): a rapid, sensitive, specific, and cost-effective point-of-care test for coronaviruses in the context of COVID-19 pandemic. Biology (Basel). 2020; 9: 182. https://doi.org/10.3390/biology9080182 PMID: 32707972

29. Sano S, Fukushi S, Yamada S, Harada S, Kinoshita H, Sugimoto S, et al. Development of an RT-LAMP assay for the rapid detection of SFTS virus Viruses. 2021; 13: 693. https://doi.org/10.3390/v13040693 PMID: 33923720

30. Paris DH, Blacksell SD, Newton PN, Day NP. Simple, rapid and sensitive detection of Orientia tsutsugamushi by loop-isothermal DNA amplification. Trans R Soc Trop Med Hyg. 2008; 102: 1239–1246. https://doi.org/10.1016/j.trstmh.2008.04.040 PMID: 18565558

31. Baek YH, Cheon HS, Park SJ, Lloren KKS, Ahn SJ, Jeong JH, et al. Simple, rapid and sensitive portable molecular diagnosis of SFTS virus using reverse transcriptional loop-mediated isothermal amplification (RT-LAMP). J Microbiol Biotechnol. 2018; 28:1928–1936. https://doi.org/10.4014/jmb.1806.06016 PMID: 30270605

32. Nagamine K, Hase T, Notomi T. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes. 2002; 16: 223–229. https://doi.org/10.1006/mcpr.2002.0415 PMID: 12144774

33. Bhat IA, Mashooq M, Kumar D, Varshney R, Rathore R. Development of probe-based real-time loop-mediated isothermal amplification for detection of Brucella. J Appl Microbiol. 2019; 126: 1332–1339. https://doi.org/10.1111/jam.13938 PMID: 29851222

34. Tanner NA, Zhang Y, Evans TC Jr. Simultaneous multiple target detection in real-time loop-mediated isothermal amplification. Biotechniques. 2012; 53: 81–89. https://doi.org/10.2144/0000113902 PMID: 23030060

35. Yoshikawa T, Fukushi S, Tani H, Fukuma A, Taniguchi S, Toda S, et al. Sensitive and specific PCR systems for detection of both Chinese and Japanese severe fever with thrombocytopenia syndrome virus strains and prediction of patient survival based on viral load. J Clin Microbiol. 2014; 52: 3325–3333. https://doi.org/10.1128/JCM.00742-14 PMID: 24989600.

36. Sui X, Zhang X, Fei D, Zhang Z, Ma M. Simultaneous rapid detection of Hantaan virus and Seoul virus using RT-LAMP in rats. PeerJ. 2019; 6: e6068. https://doi.org/10.7717/peerj.6068 PMID: 30643674

37. Jang WS, Kwak SY, May WL, Yang DJ, Nam J, Lim CS. Comparative evaluation of three dengue duo rapid test kits to detect NS1, IgM, and IgG associated with acute dengue in children in Myanmar. PLoS One. 2019; 14: e0213451. https://doi.org/10.1371/journal.pone.0213451 PMID: 30865680

38. Patel P, Abd El Wahed A, Faye O, Prüger P, Kaiser M, Thaloengsok S, et al. A field-deployable reverse transcription recombinase polymerase amplification assay for rapid detection of the chikungunya virus. PLoS Negl Trop Dis. 2016; 10: e0004953. https://doi.org/10.1371/journal.pntd.0004953 PMID: 27685649

39. Jang WS, Lim DH, Nam J, Mihn DC, Sung HW, Lim CS, et al. Development of a multiplex isothermal amplification molecular diagnosis method for on-site diagnosis of influenza. PLoS One. 2020; 15: e0238615. https://doi.org/10.1371/journal.pone.0238615 PMID: 32915821

40. FDA. COVID-19 in vitro diagnostics emergency use authorizations. [cited Dec 2020]. https://www.fda.gov/medical-evices/coronavirus-disease-2019-covid-19-emergency-use-authorizations-medical-devices/vitro-diagnostics-euas#individual-molecular.

41. Moehling TJ, Choi G, Dugan LC, Salit M, Meagher RJ. LAMP diagnostics at the point-of-care: Emerging trends and perspectives for the developer community. Expert Review of Molecular Diagnostics. 2021; 21: 43–61. https://doi.org/10.1080/14737159.2021.1873769 PMID: 33474990

42. Kim JH, Lee CS, Moon C, Kwak YG, Kim BN, Kim ES, et al. Co-Infection of scrub typhus and human granulocytic anaplasmosis in Korea, 2006. J Korean Med Sci. 2019; 34: e257. https://doi.org/10.3346/jkms.2019.34.e257 PMID: 31602827

43. Wi YM, Woo HI, Park D, Lee KH, Kang CI, Chung DR, et al. Severe fever with thrombocytopenia syndrome in patients suspected of having scrub typhus. Emerg Infect Dis. 2016; 22: 1992–1995. https://doi.org/10.3201/eid2211.160597 PMID: 27767909

44. Thi Hai Yen N, Kim C, Jeong S, Jeon K, Choi H, Ro HJ, et al. Severe fever with thrombocytopenia syndrome virus infection or mixed infection with scrub typhus in South Korea in 2000–2003. Am J Trop Med Hyg. 2019; 101: 1096–1099. https://doi.org/10.4269/ajtmh.19-0392 PMID: 31482787

45. Ra SH, Kim JY, Cha HH, Kwon JS, Lee HJ, Jeon NY, et al. Coinfection of severe fever with thrombocytopenia syndrome and scrub typhus in patients with tick-borne illness. Am J Trop Med Hyg. 2019; 101: 1259–1262. https://doi.org/10.4269/ajtmh.19-0242 PMID: 31549609

46. Yoo IY, Kim JY, Yoon YK, Huh HJ, Lee NY. Comparison between the SFTS-QS kit and the PowerChek SFTSV real-time PCR kit for the detection of severe fever with thrombocytopenia syndrome virus. Ann Lab Med. 2020; 40: 317–320. https://doi.org/10.3343/alm.2020.40.4.317 PMID: 32067431

47. Watthanaworawit W, Turner P, Turner C, Tanganuchitcharnchai A, Richards AL, Bourzac KM, et al. A prospective evaluation of real-time PCR assays for the detection of Orientia tsutsugamushi and Rickettsia spp. for early diagnosis of rickettsial infections during the acute phase of undifferentiated febrile illness. Am J Trop Med Hyg. 2013; 89: 308–310. https://doi.org/10.4269/ajtmh.12-0600 PMID: 23732256

48. Le Viet N, Laroche M, Thi Pham HL, Viet NL, Mediannikov O, Raoult D, et al. Use of eschar swabbing for the molecular diagnosis and genotyping of Orientia tsutsugamushi causing scrub typhus in Quang Nam province, Vietnam. PLoS Negl Trop Dis. 2017; 11: e0005397. https://doi.org/10.1371/journal.pntd.0005397 PMID: 28241043