Clinical Usefulness of Nested Reverse-Transcription Polymerase Chain Reaction for the Diagnosis of Severe Fever with Thrombocytopenia Syndrome

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Abstract. Severe fever with thrombocytopenia syndrome (SFTS), caused by SFTS virus (SFTSV) is an emerging tick-borne infectious disease. Few studies have assessed the clinical usefulness of nested reverse-transcription polymerase chain reaction (RT-PCR) for diagnosing SFTS. We performed conventional RT-PCR targeting the M segment, nested RT-PCR targeting M and S segments, and real-time RT-PCR targeting the S segment of SFTSV for four patients with suspected SFTS. Although conventional RT-PCR results for the first two patients were negative at admission, nested RT-PCR using the S or M targets was positive for the same samples. Likewise, in the other two patients, initial samples were confirmed positive in all three tests, but follow-up testing demonstrated negative conventional RT-PCR and positive nested RT-PCR results. Thus, delayed testing using conventional RT-PCR or real-time RT-PCR in symptomatic patients with SFTS may result in missed diagnoses, and compared with these methods, nested RT-PCR may increase the window for obtaining positive SFTSV PCR results. Meanwhile, the indirect immunofluorescence assay showed seroconversion to SFTSV antibodies in all four patients. Nested RT-PCR for SFTSV M and S segments could help diagnose SFTS in patients testing negative by conventional RT-PCR.

INTRODUCTION

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne infectious disease caused by the SFTS virus (SFTSV), which belongs to the genus *Bandavirus*, family *Phenuiviridae*, and order *Bunyavirales*.^{1,2} Severe fever with thrombocytopenia syndrome virus has a tripartite genome, with the three RNA segments denoted as small (S), medium (M), and large (L). The L segment encodes RNA-dependent RNA polymerase, the M segment the glycoproteins Gn and Gc, and the S segment the nonstructural (NS) protein and a nucleocapsid protein (N).¹ Severe fever with thrombocytopenia syndrome was first reported in South Korea in 2013 and can be a fatal infectious disease, with a mortality rate reaching 30%.³ A study in China reported that the interval required for the confirmation of the diagnosis of SFTS after symptom onset was a factor that could predict fatal outcomes of SFTSV infection; thus, timely and accurate diagnosis is important for a favorable prognosis of patients with SFTS.⁴ In four patients suspected of SFTS, based on the presence of fever and gastrointestinal symptoms after a tick bite and who tested negative for reverse-transcription polymerase chain reaction (RT-PCR), a nested RT-PCR targeting two different segments was useful for diagnosis. Moreover, seroconversion of SFTSV antibodies was documented using an immunofluorescence assay (IFA) in all the four patients. This study assessed the clinical usefulness of a nested RT-PCR for the diagnosis of SFTS.

MATERIALS AND METHODS

Case 1. A 47-year-old man was picking fruits at Kagongsan in Haenam-gun 3 weeks before admission (September 23, 2017). On the following day, he found and removed a tick on his right ankle. Five days before admission, he developed a severe headache. Three days before admission to

our hospital, he was admitted to a local hospital for symptoms of fever (~38.5°C), myalgia, chills, and loose stool. However, because his symptoms did not improve, he was transferred to our hospital. At the time of admission to our hospital, his vital signs included a blood pressure of 120/70 mm Hg, heart rate of 88 beats per minute, respiratory rate of 20 breaths per minute, and temperature of 36.6°C. Blood tests performed at the time of admission are shown in Table 1.

Case 2. A 53-year-old man residing on Bogildo Island, Jeollanamdo Province, with a history of hepatitis B in his 20s, for which he did not undergo any treatment, made occasional visits to the garden for cooking ingredients, as a chef. Three days before admission, he complained of abdominal pain and fever, and was admitted to a local hospital the day before admission to our hospital. He was then transferred because of laboratory results indicating decreased leukocyte and platelet counts and increased liver function test (LFT) levels. At the time of admission, his vital signs included a blood pressure of 120/70 mm Hg, heart rate of 56 beats per minute, respiratory rate of 20 breaths per minute, and temperature of 36.8°C. Blood tests performed at the time of admission are listed in Table 1.

Case 3. An 80-year-old woman without any underlying health conditions or participation in outdoor activities complained of frequent watery diarrhea averaging > 10 times a day, which began 3 days before admission to our hospital. She was admitted to a local hospital where she presented with decreased systolic blood pressure, and upon examination for potential sepsis, was transferred to our hospital because of bicytopenia and elevated LFT levels.

At the time of admission, her vital signs included a blood pressure of 110/70 mm Hg, heart rate of 96 beats per minute, respiratory rate of 20 breaths per minute, and temperature of 37.9°C. While administering ciprofloxacin for potential sepsis associated with colitis, SFTS was suspected and the patient was tested for SFTSV, for which the results were positive.

Case 4. A 79-year-old man on medication for hypertension had been gardening or planting trees in the countryside after retirement. He complained of fever and myalgia 5 days before admission to our hospital and visited a local hospital the day before admission, from where he was transferred because of

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TABLE 1
Severe fever with thrombocytopenia syndrome diagnostic test results at different time points in four patients admitted for fever after a tick bite

Patient	Tick bite site	Peak fever (°C)	Symptom onset/treatment (duration)	Hb/WBC/platelet (g/dL/ $10^3/\mu\text{L}/10^3/\mu\text{L}$)	AST/ALT (U/L)	CRP (mg/dL)	Sampling day	Nested RT-PCR			IFA	
								RT-PCR (M segment)	M segment	S segment	Real-time RT-PCR (S segment)	IgG
Patient 1: Male, age 47	Right ankle	38.5	October 6, 2017 Conservative treatment	15.7/1.98/109 15.9/1.88/102	44.9/23.5 76.4/53.8	— 0.12	10/11 10/12	Negative Negative	Positive Positive	Positive Positive	38.78 37.19	< 32 < 32
Patient 2: Male, age 53	No	37.1	September 9, 2018 IVI/G (September 17, 2018–September 19, 2018)	16.8/3.87/166 13.4/1.70/56	108.6/174.7 167.2/157.1	0.03 0.12	10/18 9/13	Negative Negative	Negative Positive	Negative Positive	41.05 37.42	< 32 < 32
Patient 3: Female, age 80	No	37.9	October 6, 2018 IVI/G (October 13, 2018–October 14, 2018)	13.3/2.38/78 14.3/2.25/146	150/141 101/133	0.17 0.10	9/15 9/17	Negative Negative	Negative Negative	Negative Negative	Undetermined 42.07	< 32 512
Patient 4: Male, age 79	No	37.3	July 5, 2018 IVI/G (July 12, 2018–July 13, 2018)	15.6/1.33/60 13.7/2.05/21	268.1/142.2 182.9/87	0.03 0.10	10/9 10/12	Positive Positive	Positive Positive	Positive Positive	29.35 28.57	128 256
				10.8/2.76/58	130/97.3	0.84	10/15	Negative	Positive	Positive	35.38	512
				14.4/2.87/68	315.7/103.8	0.92	7/10	Positive	Positive	Positive	31.9	< 32
				12.4/6.47/182	90.3/77.3	1.29	7/20	Negative	Positive	Positive	43.96	256
				—	—	—	7/30	Negative	Positive	Positive	41.06	> 1,024

ALT = alanine aminotransferase; AST = aspartate aminotransferase; CRP = C-reactive protein; Hb = hemoglobin; IFA = indirect immunofluorescence antibody assay; IVIG = intravenous immunoglobulin; M = medium; PCR = polymerase chain reaction; RT = reverse transcription; RT-PCR = reverse transcription polymerase chain reaction; S = small; SFTS = severe fever with thrombocytopenia syndrome; WBC = white blood cell.

suspected SFTS based on test results indicating a temperature of 38.4°C, decreased white blood cell and platelet counts, and elevated LFT levels. His vital signs included a blood pressure of 150/80 mm Hg, heart rate of 74 beats per minute, respiratory rate of 20 breaths per minute, and temperature of 37°C. He was tested upon admission because his clinical presentation indicated suspected SFTS.

PCR diagnostic methods. Viral RNA was extracted from whole blood collected from the patient on the day of admission using the Viral Gene-spin™ Viral DNA/RNA Extraction Kit (iNTRON Biotechnology, Sungnam-si, Korea). For the SFTSV-targeted RT-PCR, the SuperScript IV VILO Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania) was used to synthesize cDNA, and the AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA) and the Veriti™ 96-Well thermal cycler (Applied Biosystems) were used to perform RT-PCR targeting the SFTSV M segment.⁵ For nested RT-PCR targeting the M segment (encoding the viral envelope glycoprotein), the first set of PCR primers were designed in-house, and the MF3/MR2 primers described previously for the diagnosis of SFTS were used as the second set of PCR primers.⁶ The nested RT-PCR targeting the S segment (encoding the viral nucleocapsid and NS protein) was performed using previously reported primers.⁷ For the real-time RT-PCR, previously published articles were referenced for the synthesis of primers and the probe targeting the S segment.⁸ The PCR primers and probe used in this study are described in Table 2.

After purifying the 560-bp M segment and 346-bp S segment RT-PCR products, the sequences were determined and analyzed using the BlastN (Bethesda, MD) network service from the National Center for Biotechnology Information (National Institutes of Health). Moreover, the M and S segment sequences obtained from the GenBank database and patient samples were used to construct a phylogenetic tree with the ClustalX (Ver 2.0; www.clustal.org/) and Tree Explorer programs (DNASTAR, Madison, WI). To increase the reliability of this tree, a 1,000-iteration bootstrap analysis was used.

Indirect immunofluorescence antibody assay diagnostic method. To assess an indirect IFA for the diagnosis of SFTS, Vero E6 cells were infected with SFTSV, and the infected cells were inoculated and fixed with 80% acetone on Teflon-coated well slides to prepare the SFTS antigen slide. After stepwise 2-fold dilution of serum from 1:32, 20 μL of diluted serum was dropped into the slide wells and allowed to react with the viral antigen. The test slides were incubated for 30 minutes at 37°C with constant humidity. After washing with phosphate-buffered saline (PBS) and distilled water, secondary antibodies diluted 1:400 (fluorescein isothiocyanate-conjugated anti-human IgM and IgG; MP Biomedicals, Illkirch, France) were applied to each slide well and allowed to react for 30 minutes. Subsequently, the slide was washed again with PBS and distilled water. The slide was dried and an Antifade mounting medium (Vector Laboratories, Burlingame, CA) was added dropwise. Then, a cover slip was mounted on the slide and the slide was observed with a fluorescence microscope under 400 $\times$ magnification.^{9,10} The final serum dilution factor that showed specific fluorescence was defined as the antibody titer.

RESULTS

In patient 1, SFTSV-targeting conventional RT-PCR was performed because the patient had a history of a tick bite.

TABLE 2

Reverse-transcription polymerase chain reaction, nested RT-PCR, and real-time RT-PCR primers and probes used to assess the SFTS diagnosis

Target gene	PCR	Primer name	Sequences (5'-3')	Product size (bp)
M segment	RT-PCR	SFTS-F(=MF3)	GATGAGATGGTCCATGCTGATTCTAA	560
		SFTS-R(=MR2)	CTCATGGGGTGGGAATGTCCTCAC	
	Nested RT-PCR (first PCR)	SFTS-M 1 st -F	TCATCCTGACYTATTTGCAATWG	640
		SFTS-M 1 st -R	TAAGTYACACTCACACCCCTTGAA	
S segment	Nested RT-PCR (second PCR)	SFTS-F(=MF3)	GATGAGATGGTCCATGCTGATTCTAA	560
		SFTS-R(=MR2)	CTCATGGGGTGGGAATGTCCTCAC	
	Nested RT-PCR (first PCR)	SFTS-S-NP-2F	CATCATTGTCTTTGCCCTGA	461
		SFTS-S-NP-2R	AGAAGACAGAGTTTACAGCA	
	Nested RT-PCR (second PCR)	SFTS-S-N2F	AAYAAGATCGTCAAGGCATCA	346
		SFTS-S-N2R	TAGTCTTGTTGAAGGCATCTT	
	Real-time RT-PCR	SFTS-SQ-F	ACCTCTTTGACCCTGAGTTWGACA	120
		SFTS-SQ-R	CTGAAGGAGACAGGTGGAGATGA	
		SFTS-SQ-P	[FAM]-TGCCTTGACGATCTTA[NFQ-MGB]	

M = medium; PCR = polymerase chain reaction; RT = reverse transcription; S = small; SFTS = severe fever with thrombocytopenia syndrome.

Although the results were negative, because of the presentation of clinical feature characteristic of SFTS, real-time RT-PCR and nested RT-PCR were additionally performed (Table 1). The result of real-time RT-PCR with an S segment target was negative with a threshold cycle (Ct) value of 38.78, whereas nested RT-PCR results for the M and S segments were positive, in addition to an IFA test performed 1 week later, which confirmed the seroconversion of IgG titers.

Patient 2 also presented clinical features of SFTS and was thus tested upon admission. Three types of PCR tests were run using a sample, out of which conventional RT-PCR results were negative, although results of real-time RT-PCR were positive at Ct value of 37.42, as were the results for nested RT-PCR. The three PCR tests were repeated on days 2 and 4 following the admission; the results for all of them were negative. Seroconversion of IFA antibody titers, however, was confirmed.

In patient 3, SFTS was suspected during the administration of ciprofloxacin for potential sepsis associated with colitis. The three PCR tests were performed, all of which gave positive results. Intravenous immunoglobulin was administered on the 4th day of admission, after which the nested RT-PCR results were positive with a real-time RT-PCR Ct value of 35.38. Conventional RT-PCR result, however, was negative.

The three PCR tests were performed on patient 4, who was also suspected of having an SFTS infection, and the results were positive. After 10 days, nested RT-PCR results on both targets remained positive, with a real-time RT-PCR Ct value over 40. Although conventional RT-PCR results were negative on days 10 and 20 following admission, nested RT-PCR tests revealed positive results in both targets 20 days after admission.

The M and S segments nested RT-PCRs using the blood samples from the four patients on the day of admission both showed positive results. The sequence analysis of the positive RT-PCR products revealed the presence of SFTSV in the specimens from the four patients. A phylogenetic tree was constructed based on the nucleotide sequences of partial M and S segments obtained from the four patients and from various SFTSV in GenBank (Figure 1). The phylogenetic trees showed that both M and S segment partial sequences from patient 1 (2017-770) and patient 4 (2018-638) formed a cluster with Japanese 3 (J3) clade of SFTSV strains. Moreover, the M and S segment partial sequences from patient 2 (2018-913) and patient 3 (2018-980) formed a cluster with Japanese 1 (J1) clade of SFTSV strains.

Gene homology analysis with the M segment partial sequences of the nested RT-PCR products from patient 1 (2017-770), patient 2 (2018-913), patient 3 (2018-980), and patient 4 (2018-638) showed 99.4% sequence similarity (532/535) with the SFTSV isolate KADGH from a Korean patient (accession no. KU507548.1), 99.3% sequence similarity (538/542) with the SFTSV isolate 16MS347 from a Korean patient (accession no. MG737179.1), 99.1% sequence similarity (538/543) with the SFTSV isolate 15MS132 from a Korean patient (accession no. MG737151.1), and 99.8% sequence similarity (536/537) with the SFTSV isolate ZJZSHS-LWL/China/08/2014 from a Chinese patient (accession no. KR017863.1), respectively. Moreover, gene homology analysis with the S segment partial sequences of the nested RT-PCR products from patient 1 (2017-770), patient 2 (2018-913), patient 3 (2018-980), and patient 4 (2018-638) showed 99.7% sequence similarity (338/339) with the SFTSV isolate KADGH from a Korean patient (accession no. KU507553.1), 99.4% sequence similarity (332/334) with the SFTSV isolate 16MS347 from a Korean patient (accession no. MG737287.1), 100% sequence similarity (340/340) with the SFTSV isolate 15KS23 from a Korean patient (accession no. MG737240.1), and 99.1% sequence similarity (331/334) with the SFTSV isolate MS39 from a Korean patient (accession no. MF094818.1), respectively.

DISCUSSION

Severe fever with thrombocytopenia syndrome is a potentially fatal tick-borne infectious disease with reported mortality rates of 32.6% in South Korea¹¹ and 31% in Japan.^{12,13}

A study in China reported that the confirmation interval for the diagnosis of SFTS after symptom onset was a factor that could help predict the severity of SFTSV infection⁴ and that admission after symptom onset of patients with severe SFTS was delayed compared with the timing of admission of patients with mild SFTS, which demonstrated the need for timely diagnosis of SFTS. RT-PCR and real-time RT-PCR are commonly used for the diagnosis of SFTS; however, no studies have assessed the clinical usefulness of nested RT-PCR.

A study of 108 Japanese patients suspected of having SFTS reported that conventional one-step RT-PCR and quantitative one-step RT-PCR had completely concordant results, indicating that quantitative one-step RT-PCR was as clinically useful as conventional one-step RT-PCR.¹³

M segment

S segment

FIGURE 1. A phylogenetic tree based on partial sequences of M segment (477 bp) and S segment (321 bp) from GenBank and whole blood from four patients suspected of having severe fever with thrombocytopenia syndrome virus (SFTSV) infection. Scale bars indicate 0.01 base substitutions per site. GenBank accession numbers are shown in the tree.

However, quantitative RT-PCR and serological testing using IgM antibodies may not detect all cases of SFTS.¹⁴ In other words, because detecting all SFTS cases using conventional or real-time RT-PCR may be difficult, highly sensitive diagnostic methods are urgently needed.

To the best of our knowledge, no studies have assessed the clinical usefulness of nested RT-PCR in actual patients with SFTS.

The first two cases had negative initial conventional RT-PCR results, but had positive nested RT-PCR results using S or M targets using the same sample. In contrast, in patients 3 and 4, positive results in all three tests were obtained using the initial sample, although positive nested RT-PCR results and negative conventional RT-PCR results were obtained during the follow-up tests. In these patients, real-time RT-PCR also demonstrated elevated Ct values, which suggested that a delay in testing symptomatic patients with SFTS may result in missed diagnoses if tested with conventional RT-PCR or real-time RT-PCR. Such results were indicative of nested RT-PCR providing a wider window to obtain positive SFTSV PCR results compared with conventional RT-PCR or real-time RT-PCR. Immunofluorescence antibody assay testing also confirmed seroconversion in all the four patients, which demonstrated the clinical practicality of nested RT-PCR. Moreover, there have been reports of differences in the virulence of Japanese and Chinese strains of SFTSV in spotted doves,¹⁵ which may

be beneficial for clinical use, if presented as additional data on the viral clade of SFTSV in nested PCR.

Nested PCR method is known to be 100 times more sensitive than performing conventional PCR, but cross-contaminations and carry over-contaminations may occur. Therefore, caution is required when performing nested PCR for clinical diagnosis not to reduce the accuracy of the test because of false positive.^{16,17}

In cases of clinically suspected SFTS that test negative by conventional RT-PCR at an early stage of SFTS symptoms, nested RT-PCR and follow-up PCR tests may be useful for the accurate diagnosis of SFTS. Therefore, a nested RT-PCR targeting two different segments, such as the SFTSV M and S segments, may help diagnose patients with a strong clinical suspicion of SFTS, even when the conventional RT-PCR results are negative.

In conclusion, the results of this study indicated that nested RT-PCR targeting two different segments could help diagnose SFTS in patients with suspected SFTS infections, even when the conventional RT-PCR showed negative results.

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