Rapid Diagnosis of Tick-Borne Illnesses by Use of One-Step Isothermal Nucleic Acid Amplification and Bio-Optical Sensor Detection

Ji Yeun Kim,[1†] Bonhan Koo,[2†] Choong Eun Jin,[2] Min Chul Kim,[1] Yong Pil Chong,[1] Sang-Oh Lee,[1] Sang-Ho Choi,[1] Yang Soo Kim,[1] Jun Hee Woo,[1] Yong Shin,[2*] and Sung-Han Kim[1*]

BACKGROUND: Scrub typhus and severe fever with thrombocytopenia syndrome (SFTS) are the most common tick-borne illnesses in South Korea. Early differentiation of SFTS from scrub typhus in emergency departments is essential but difficult because of their overlapping epidemiology, shared risk factors, and similar clinical manifestations.

METHODS: We compared the diagnostic performance of one-step isothermal nucleic acid amplification with bio-optical sensor detection (iNAD) under isothermal conditions, which is rapid (20–30 min), with that of real-time PCR, in patients with a confirmed tick-borne illness. Fifteen patients with confirmed SFTS who provided a total of 15 initial blood samples and 5 follow-up blood samples, and 21 patients with confirmed scrub typhus, were evaluated.

RESULTS: The clinical sensitivity of iNAD (100%; 95% CI, 83–100) for SFTS was significantly higher than that of real-time PCR (75%; 95% CI, 51–91; $P = 0.047$), while its clinical specificity (86%; 95% CI, 65–97) was similar to that of real-time PCR (95%; 95% CI, 77–99; $P = 0.61$). The clinical sensitivity of iNAD for scrub typhus (100%; 95% CI, 81–100) was significantly higher than that of real-time PCR for scrub typhus (67%; 95% CI, 43–85; $P = 0.009$), while its clinical specificity (90%; 95% CI, 67–98) was similar to that of real-time PCR (95%; 95% CI, 73–100; $P > 0.99$).

CONCLUSIONS: iNAD is a valuable, rapid method of detecting SFTS virus and *Orientia tsutsugamushi* with high clinical sensitivity and specificity.

Severe fever with thrombocytopenia syndrome (SFTS)[3] and scrub typhus are common tick-borne infectious diseases in eastern Asia, especially Korea, China, and Japan *(1, 2)*. SFTS is caused by SFTS virus (SFTSV), a novel *Phlebovirus* in the family *Bunyaviridae*, which was first identified in China in 2009 *(3)*, and subsequently found in Korea and Japan *(4, 5)*. In China, the mortality rate of SFTS is about 12% *(6)*. In Korea, the incidence of SFTS increased from 36 cases in 2013 to 79 cases in 2015, and the overall mortality rate was higher than that in China, with 54 deaths among 170 confirmed cases (32%) *(7)*. Scrub typhus is caused by *Orientia tsutsugamushi*, classified in the family *Rickettsiaceae (8)*. It is prevalent in the Asia-Pacific region, where about 1 million cases are reported annually *(9)*. In Korea, 5000–6000 cases have been reported annually since 2005 *(7)*, and the prevalence has increased, with 9513 confirmed cases reported in 2015 *(7)*. However, the clinical presentations of scrub typhus substantially overlap with those of SFTS. Therefore, laboratory tests to differentiate early between SFTS and scrub typhus are urgently needed to ensure specific antimicrobial treatment for scrub typhus and to institute appropriate precautions for SFTS because of its potential transmission to healthcare workers *(10)*.

The diagnosis of scrub typhus generally relies on serologic tests, especially indirect immunofluorescence assays (IFA), which can detect a 4-fold increase in antibody titer between paired sera *(11, 12)*. However, this serologic test requires paired serum samples and cannot be used in the acute phase of the disease. Real-time PCR has been proposed for early clinical diagnosis of scrub typhus *(13, 14)*, but the samples assayed in these studies were buffy coats, which required technical expertise for their preparation. Because *O. tsutsugamushi* is an obligate

[1] Departments of Infectious Diseases, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea; [2] Convergence Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea.

* Address correspondence to: S.-H. K. at Department of Infectious Diseases, Asan Medical Center, University of Ulsan College of Medicine, 88 Olympic-ro-43-gil, Songpa-gu, Seoul, 05505, South Korea. Fax +82-2-3010-6970; e-mail kimsunghanmd@hotmail.com; or Y. S. at Department of Convergence Medicine, Asan Medical Center, University of Ulsan College of Medicine, 88 Olympic-ro-43-gil, Songpa-gu, Seoul, 05505, South Korea. Fax +82-2-3010-4193; e-mail shinyongno1@gmail.com.

† These authors equally contributed to the work.

Received July 31, 2017; accepted October 30, 2017.
Previously published online at DOI: 10.1373/clinchem.2017.280230

[3] Nonstandard abbreviations: SFTS, severe fever with thrombocytopenia syndrome; SFTSV, SFTS virus; IFA, immunofluorescence assays; RT, reverse transcription; iNAD, isothermal nucleic acid amplification procedure with bio-optical sensor detection; SMR, silicon microring resonator; RPA, recombinase polymerase amplification; cDNA, complementary DNA.

intracellular parasite *(1)*, bacterial loads in sera are expected to be low. Therefore, the clinical sensitivity of real-time PCR for detecting *O. tsutsugamushi* by use of serum samples remains insufficient *(13, 15)*. Real-time reverse transcription (RT)-PCR for SFTSV has been developed recently for differential diagnosis of SFTS *(16, 17)*, but neither has it been standardized nor is it commercially available. In sum, serologic diagnosis of scrub typhus is of limited use in clinical practice, and molecular diagnosis has suboptimal clinical sensitivity, especially for scrub typhus. In addition, both conventional tests take several hours, so a more rapid and sensitive test to differentiate common tick-borne illnesses is needed.

In this study, we developed a 1-step isothermal nucleic acid amplification procedure with bio-optical sensor detection (iNAD) that has high sensitivity, specificity, and is rapid (20–30 min) under isothermal conditions for both DNA and RNA analysis. This assay is based on a combination of 2 techniques, isothermal solid-phase DNA amplification and detection (iSAD) for DNA analysis and isothermal 1-step RNA amplification and detection (iROAD) for RNA analysis *(18, 19)*. Our goal was to compare the diagnostic performance of iNAD with that of real-time PCR with use of plasma specimens from patients with confirmed tick-borne illnesses in South Korea.

Methods

PATIENTS AND SPECIMEN COLLECTION AND PROCESSING

We prospectively enrolled adult patients with suspected tick-borne illness in Asan Medical Center, a 2700-bed tertiary hospital in Seoul, South Korea, between 2015 and 2016, and collected blood samples from these patients. About 8 mL of blood was collected in EDTA tubes from the patients, and plasma was obtained by centrifugation at 1200*g* for 10 min. Each 500 μL of plasma was aliquoted into 1.5 mL of microcentrifuge tubes and stored at −80 °C until testing. One vial of plasma (500 μL) was sent to the Korea Centers for Disease Control to confirm infection of SFTSV by detecting viral RNA by RT-PCR, by use of a DiaStar 2X OneStep RT-PCR PreMix kit (SolGent) as previously described *(10)*. The remaining plasma was stored at −80 °C for further use.

A diagnosis of scrub typhus was established when either a single positive result from IFA (SD. Bioline Tsutsugamushi Assay; Standard Diagnostics), or a ≥1:640 or 4-fold rise of IFA titer in successive samples was documented *(20)*. The stored plasma samples from the patients confirmed with SFTS and scrub typhus were thawed to compare the diagnostic performance of iNAD with that of real-time PCR. On the basis of previous studies *(13–17)*, we initially expected that the clinical sensitivity of real-time PCR for scrub typhus will not be high in confirmed patients with scrub typhus, while the clinical sensitivity of real-time PCR for SFTSV will be high in patients with SFTS. Therefore, we included the recovery phase plasma samples in case of patients with SFTS so that we could evaluate the ability of iNAD to detect a very low viral load of SFTSV. The final classification of the patients' plasma specimens was blinded to the research laboratory personnel. Both DNA and RNA were simultaneously extracted from each plasma sample. The extracted DNA for scrub typhus and RNA for SFTS were used for real-time PCR of SFTSV and scrub typhus, and iNAD of SFTSV and scrub typhus. True positive or negative results of the real-time PCR and iNAD were determined by the final diagnosis of SFTS confirmed by RT-PCR of the Korea Centers for Disease Control, and scrub typhus by IFA results for scrub typhus. The sample processing flow chart is shown in Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol64/issue3. The Institutional Review Board of Asan Medical Center approved the study protocol, and informed consent was obtained from all participants.

DESIGN OF PRIMERS AND PROBES

Primers and probes for SFTSV were designed by use of the SFTSV M and S segments. To detect *O. tsutsugamushi*, we used the 56-kDa type-specific antigen gene, which encodes the primary immunogen located on the outer membrane *(21, 22)* commonly used in conventional as well as real-time PCR *(23, 24)*. To identify highly conserved regions, the following sequences were aligned by use of the ClustalW program *(25)*: M segments of SFTSV (GenBank accession numbers: NC018138, KR698332, KR017863, AB985654, KF887440, KJ597824, KF358692, KC473541), S segments of SFTSV (Gen Bank accession numbers: NC018137, KR612087, KR612074, KR612073, KR612072, KR698319, KT254589, KU738910, KR017826), and the 56-kDa type-specific antigen gene of *O. tsutsugamushi* (GenBank accession numbers: KJ742368, KJ868218, KF523362, JQ898387, HQ660214, HQ660200, HQ731680, GU446620). The Primer3 program was used to design the primers and probes for real-time PCR *(26)*. The specificity of each primer and probe was checked by BLAST search against the NCBI database. The primers and probes for iNAD were designed manually, based on the sequences of the primers and probes for real-time PCR. The primers and probes used in this study are shown in Table 1.

EXTRACTIONS OF RNA AND DNA AND PREPARATION OF STANDARD CONTROLS

Viral RNA was extracted with a QIAamp Viral RNA Kit (Qiagen Inc.), and genomic DNA was extracted with a QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions.

Table 1. Primer and probe sequences used.

Assay	Name (accession #)	Start	Sequence	Modification
Real-time PCR	SFTS SF (NC018137)	116	CGAGAGAGCTGGCCTATGAA	
	SFTS SR	263	TTCCCTGATGCCTTGACGAT	
	SFTS SP	216	TGTCTTTGCCCTGACTCGAGGCA	5′ FAM, 3′ BHQ1
	SFTS MF (NC018138)	316	ATGCTTGTCGTGAAGAAGGC	
	SFTS MR	446	CTAGACTTCCCACTGCCACA	
	SFTS MP	400	ACTTTTGATGGATACGTAGGCTGGGGC	5′Cy5, 3′ BHQ2
	ACBT F (NC000007.14)	1670	ACTAACACTGGCTCGTGTGA	
	ACBT R	1774	CTTGGGATGGGGAGTCTGTT	
	ACBT P	1700	AGGCTGGTGTAAAGCGGCCTTGG	5′ HEX, 3′BHQ1
	STF (KJ742368)	781	GCAGCAGCTGTTAGGCTTTT	
	STR	919	TTGCAGTCACCTTCACCTTG	
	STP	850	CAGCGTCATGCAGGAATTAGGAAAGCCA	5′FAM, 3′BHQ1
iNAD	iNAD-SFTS F (NC018137)	544	GGAGGCCTACTCTCTGTGGCAAGATGCCTTCA	5′ AmMC12
	iNAD-SFTS R	675	GGCCTTCAGCCACTTTACCCGAACATCATTGG	
	iNAD-ST F (KJ742368)	778	GCAGCAGCAGCTGTTAGGCTTTTAAATGGCAATG	5′ AmMC12
	iNAD-ST R	911	GCTGCTTGCAGTCACCTTCACCTTGATTCTTTG	

To prepare SFTS viral RNA transcript controls, fragments containing the target regions of the assays were amplified with primers containing the T7 promoter sequence on the antisense strand. The amplicons were then transcribed in vitro by use of a MEGAscriptT7 Transcription Kit (AmbionLife Technologies). Synthetic RNA transcripts were purified with a MEGAclear Kit (Ambion) and quantified with a Nanodrop spectrophotometer (Thermo Scientific).

Plasmids containing the amplified regions of the M and S segments of SFTSV and the 56-kDa antigen gene of *O. tsutsugamushi* were generated by ligation into pGEM-T Easy Vector (Promega) and transferred into *Escherichia coli* JM109. *E. coli* strains were stored in glycerol at −80 °C.

TAQMAN PROBE-BASED REAL-TIME PCR

Multiplex real-time RT-PCR assays with Taqman probe to detect SFTSV were performed by use of a LightCycler Multiplex RNA Virus Master (Roche), in 20 μL of reaction mixtures containing 4 μL of 5X master mix, 0.1 μL of 200X enzyme mix, 4 μL primer and probe mix, and 5 μL of extracted RNA or synthetic RNA. The primer and probe mix consisted of 250 nmol/L S segment, M segment, and β-actin primers, 250 nmol/L S segment probe, and 125 nmol/L M segment and β-actin probes. RT-PCR amplification was performed with a LightCycler 96 system (Roche) with the following conditions: reverse transcription at 50 °C for 10 min, preincubation at 95 °C for 10 min, followed by 45 cycles of 2-step amplification (95 °C for 5 s and 56 °C for 30 s). To generate a calibration curve, serial dilutions from 10^7 to 10^1 copies/μl of synthetic RNA were assayed in 5 independent sets of reactions. To detect *O. tsutsugamushi*, Taqman probe-based real-time PCR was performed with FastStart Essential DNA Probes Master (Roche) in 20 μL of reaction mixtures containing 10 μL of 2X master mix, 250 nmol/L of ST primers, 100 nmol/L of ST probe, and 5 μL of extracted DNA or control DNA. PCR amplification was performed with use of a LightCycler 96 system (Roche) in the following conditions: preincubation at 95 °C for 10 min followed by 45 cycles of 2-step amplification (95 °C for 10 s and 60 °C for 30 s). A calibration curve for quantification of *O. tsutsugamushi* was generated with serial dilutions of control DNA from 10^6 to 10^1 copies/μL. All experiments were run in duplicate, and positive and negative controls were included in each assay. The SYBR Green–based real-time PCR method is described in the Methods file in the online Data Supplement.

iNAD

For the iNAD assay, detailed protocols combining silicon microring resonators (SMRs) and recombinase polymerase amplification (RPA) were used *(18, 19)*. The preparation and operation of SMR biosensor are described in the Methods file in the online Data Supplement. The SMR sensor is a refractive index-based optical sensor that changes resonance properties in response to binding between target and ligand; it provides highly sensitive, label-free, real-time detection of biomolecules near the sensor *(27, 28)*. RPA generates a complex of primer and recombinase that extends the DNA and eliminates the need for a nucleic acid polymerase and repetitive cycles

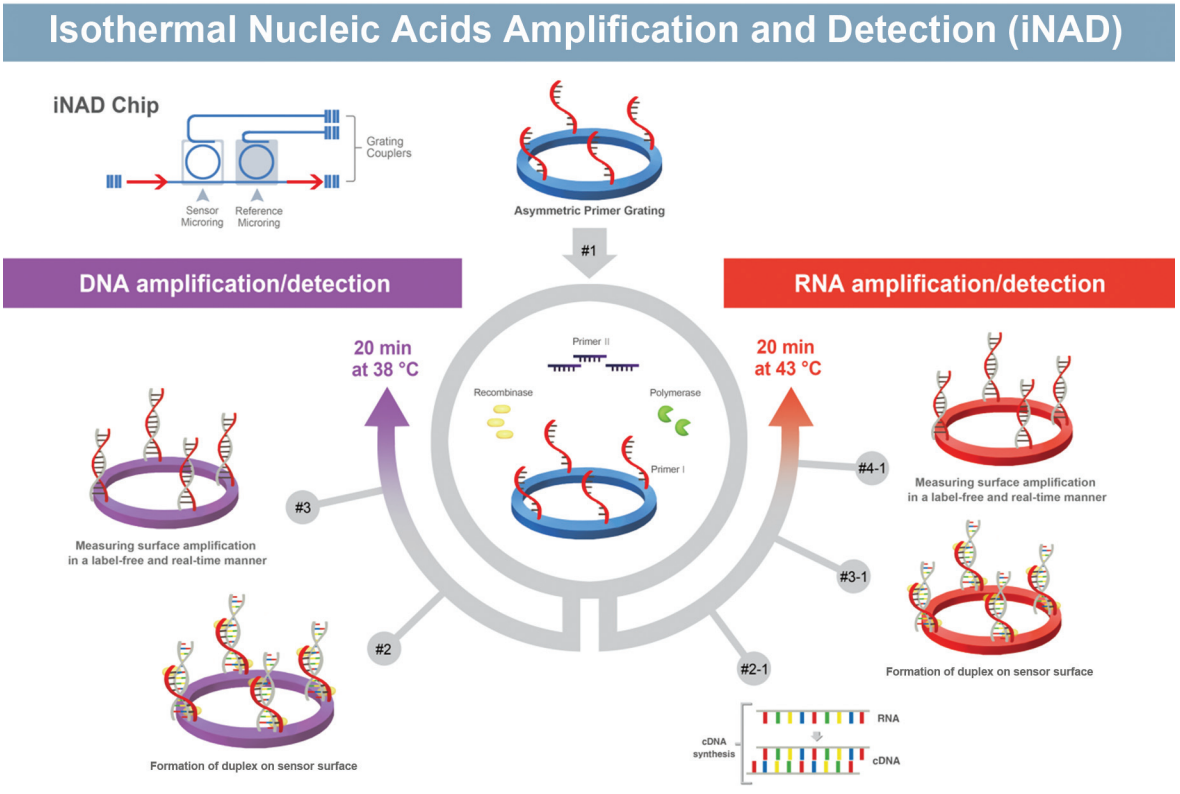

Fig. 1. Schematic representation of the principle of one-step isothermal nucleic acid amplification with bio-optical sensor detection (iNAD).

The iNAD chip consisted of a sensor microring and reference microring. Each microring had a dedicated output waveguide (grating couplers). To operate the iNAD, the forward primers were immobilized on the iNAD chip (#1). Then, a mixture of RPA and the reverse primer was added to the chip to detect DNA (left) or RNA (right). For RNA amplification and detection (right), the complimentary DNA (cDNA) was synthesized from the RNA during the reaction (#2–1). This cDNA recognizes the immobilized forward primer on the chip surface, and the wavelength shift (#4–1) due to formation of a duplex of the cDNA and primer (#3–1) is measured in a label-free and real-time manner. For DNA amplification and detection (left), the target DNA recognizes the immobilized forward primer on the chip surface. A duplex is formed between the cDNA and primer (#2) and the resulting wavelength shift (#3) is measured in a label-free and real-time manner.

(29). Fig. 1 illustrates the iNAD assay designed for clinical detection of bacterial DNA and viral RNA extracted from the plasma of patients with either SFTS or scrub typhus by use of QIAamp DNA and RNA kits, respectively. Following the extraction of DNA or RNA, the target region is amplified by an isothermal-based asymmetric nucleic acid amplification method with use of either RPA for DNA detection (Fig. 1, left) or RPA-RT reagents generating complementary DNA (cDNA) from RNA templates for RNA detection (Fig. 1, right). For the iNAD assay, one primer was grafted covalently to the optical sensor surface and the other primer was in solution, while the temperature was kept constant at 38 °C (for DNA) or 43 °C (for RNA). In the case of RNA, RPA-RT components contained a reverse transcriptase to covert RNA templates into DNA. Thus, cDNA was obtained from the viral RNA template with RPA-RT reaction. As a result, the cDNA was hybridized with the immobilized primer on the amine-modified SMR surface, and then the target amplification commenced from the RPA-RT mixture. The amplified targets were detected with the grafted primer on the sensor surface in a label-free and real-time manner (Fig. 1). Subsequently, the iNAD assay detected the RNA-based SFTS and DNA-based *O. tsutsugamushi* targets in 20 min by measuring resonance wavelength shifts.

STATISTICAL ANALYSIS

The Student *t*-test and the χ^2 test were used to compare the baseline clinical characteristics of SFTS and scrub typhus patients. The performances of real-time PCR and iNAD were compared by use of the χ^2 test. IBM SPSS statistics for Windows, version 22.0 (IBM Corp.), was

Table 2. Baseline clinical characteristics of 15 patients with severe fever with thrombocytopenia syndrome (SFTF) and 21 patients with scrub typhus.

	SFTS (n = 15)	Scrub typhus (n = 21)	P value
Age, years, mean ± SD	61 ± 7	67 ± 14	0.13
Male sex	10	10	0.26
Season (months)			
Spring–Summer (March–August)	6	1	0.01
Fall (September–November)	9	20	
Eschar	4	16	0.005
Clinical characteristics			
Fever	15	21	>0.99
Skin rash	1	14	<0.001
Headache	7	6	0.27
Altered mental status	8	2	0.007
Underlying disease			
Previous healthy	4	12	0.07
Diabetes	3	5	>0.99
Solid tumor	0	2	0.50
Chronic liver disease	0	2	0.50
Chronic kidney disease	0	1	>0.99
Immunosuppressive condition[a]	0	5	0.06
Leukocytosis (WBC > 10 000/mm^3)	0	6	0.03
Leukopenia (WBC < 4000/mm^3)	14	4	<0.001
Thrombocytopenia (Platelet < 150 × 10^3/mm^3)	15	15	0.03
Clinical course			
ICU admission	6	2	0.04
In-hospital mortality	1	0	0.42
Treatment			
Doxycycline	11	21	0.06
Ribavirin	10	1	<0.001

SFTS, severe fever with thrombocytopenia syndrome; WBC, white blood cell.
Data are number of patients unless otherwise indicated.
[a] Defined as patients with underlying diseases, such as malignancy, liver cirrhosis, chronic renal failure, and patients receiving immunosuppressive treatment.

used for statistical analysis. Confidence intervals at the 95% level ($P \leq 0.05$) were considered in all cases.

Results

CLINICAL CHARACTERISTICS OF PATIENTS

We prospectively recruited 158 patients with febrile illness from May 2015 to November 2016. Of these patients, 15 (9%) were confirmed with SFTS by real-time RT-PCR and 21 (13%) were diagnosed with scrub typhus by a ≥1:640 or 4-fold rise in IFA antibody titer. While SFTS was found over the entire warm season between spring and fall, scrub typhus was mainly encountered in the fall (95%, Table 2). Eschars were more common in patients with scrub typhus (16/21) than in patients with SFTS (4/15, $P = 0.005$). Leukopenia (WBC < 4000/mm^3) was more frequent in patients with SFTS (14/15) than in patients with scrub typhus (4/21; $P < 0.001$), and thrombocytopenia (platelet < 150 × 10^3/mm^3) was also more common in the patients with SFTS (15/15) than in the patients with scrub typhus (15/21; $P = 0.03$). The detailed demographic data and clinical characteristics are shown in Table 2.

DEVELOPMENT OF TAQMAN PROBE-BASED REAL-TIME PCR AND INAD FOR SFTS AND SCRUB TYPHUS

Calibration curves of Taqman probe-based real-time PCR for SFTSV and *O. tsutsugamushi* were obtained

by plotting Ct values against copy numbers of synthetic transcript RNAs and control DNAs. The amplification plots (Fig. 2 in the online Data Supplement) had excellent linearity (M segment of SFTS, $R^2 = 0.9954$; S segment of SFTS, $R^2 = 0.9942$; scrub typhus, $R^2 = 0.9995$; Fig. 2 left). The detection limit for SFTSV was 10 copies per PCR reaction, and for *O. tsutsugamushi* was also 10 copies per PCR reaction. The Ct values of the serum samples are shown as dots in Fig. 2, right.

A calibration curve of iNAD for SFTSV was obtained by plotting resonance wavelength shifts (picometer) values against copy numbers of synthetic transcript RNAs or control DNAs. The R^2 correlation coefficients were 0.9717 for SFTSV and 0.9786 for *O. tsutsugamushi* (Fig. 3, left). The limit of detection for SFTS was 22 copies per reaction; for scrub typhus, 15 copies per reaction. The limits of detection of iNAD for SFTSV and *O. tsutsugamushi* were comparable to those of Taqman probe-based real-time PCR. Fig. 3, right, presents the resonance wavelength shifts of all the serum samples analyzed by iNAD.

To compare the limit of detection of iNAD with that of a different type of real-time PCR assay, a SYBR Green–based real-time PCR assay with a single primer pair was conducted (Fig. 3 in the online Data Supplement). The limit of detection for SFTSV was 220 copies per reaction, and the limit of detection for *O. tsutsugamushi* was 150 copies per reaction.

DIAGNOSTIC PERFORMANCE OF TAQMAN PROBE-BASED REAL-TIME PCR AND iNAD FOR SFTS AND SCRUB TYPHUS

In the acute phase of SFTS, the clinical sensitivities of Taqman probe-based real-time PCR and iNAD were both 100% (95% CI, 75–100). However, when samples obtained from 5 patients during the recovering phase of SFTS were included in the final analysis, the clinical sensitivity of iNAD (100%) for SFTSV was significantly higher than that of Taqman probe-based real-time PCR (75%, $P = 0.047$, Table 2). However, the clinical specificity of iNAD for SFTSV (86%) was not significantly different than that of Taqman probe-based real-time PCR (95%, $P = 0.61$). Table 1 in the online Data Supplement shows the detailed results for Taqman probe-based real-time PCR and iNAD for SFTSV- and *O. tsutsugamushi*-specific primers in the 15 patients with SFTS.

The clinical sensitivity of iNAD for *O. tsutsugamushi* (100%) was significantly higher than that of Taqman probe-based real-time PCR (67%, $P = 0.009$, Table 3), while the clinical specificity of iNAD for scrub typhus (90%) was similar to that of Taqman probe-based real-time PCR (95%, $P > 0.99$, Table 3). Table 2 in the online Data Supplement shows the results of Taqman probe-based real-time PCR and iNAD for SFTSV- and *O. tsutsugamushi*-specific primers in the 21 patients with scrub typhus.

Discussion

Scrub typhus was mostly prevalent in the fall between September and November (Table 2), which could be explained by the fact that humans are infected with scrub typhus by bites from the larvae of trombiculid mites, which hatch and suck body fluid in the fall *(1, 7)*. Although SFTS was prevalent during the entire hot season, 60% of the SFTS cases in this study occurred between September and November (Table 2). Therefore, the endemic season of SFTS overlaps with that of scrub typhus. Furthermore, the geographical distribution of outdoor activities that carry the risk of exposure to ticks, as well as the resulting clinical manifestations, substantially overlaps for SFTS and scrub typhus *(30, 31)*. Therefore, early differentiation between SFTS and scrub typhus, both of which are transmitted by ticks and can be fatal, remains challenging. We have developed a procedure, named iNAD, for detecting SFTSV and *O. tsutsugamushi* in sera and have shown that its clinical sensitivities for diagnosing SFTS and scrub typhus are higher than those of Taqman-based real-time PCR, without a significant loss of diagnostic specificity (Table 3).

In this study, the detection limits of the Taqman probe-based real-time PCR assays were slightly higher than those of iNAD. The limits of detection of our real-time PCR assay for SFTS viral RNA and scrub typhus bacterial DNA are consistent with previous reports in which the limits of detection for real-time PCR were 10 copies/μl of viral RNA *(16, 17)* and 10 copies/reaction for SFTS *(14)*. However, the real-time PCR assays developed in previous studies *(14, 16, 32)* used multiplex primers and probes to improve the analytical sensitivity and specificity of the Taqman probe assay. Our iNAD does not use any probes or multiplex primers, just a single primer pair, and nearly achieved the limits of detection of the Taqman probe-based assay. In addition, the methodological difference between SYBR Green and Taqman probes for real-time PCR must be considered *(33)*. When we conducted SYBR Green–based real-time PCR with a single primer pair, the limit of detection of SFTS was 220 copies per reaction, and that of scrub typhus was 150 copies per reaction (Fig. 3 in the online Data Supplement). This means that the iNAD is more analytically sensitive than SYBR Green–based real-time PCR with use of the same single primer pair, and its analytic sensitivity is comparable to that of Taqman probe-based real-time PCR. Additionally, the present iNAD could reduce costs since it uses a single primer set and no probes.

Both real-time RT-PCR and iNAD had 100% clinical sensitivity in the 15 SFTS sera collected over 1–3 days of hospitalization, whereas real-time RT-PCR gave

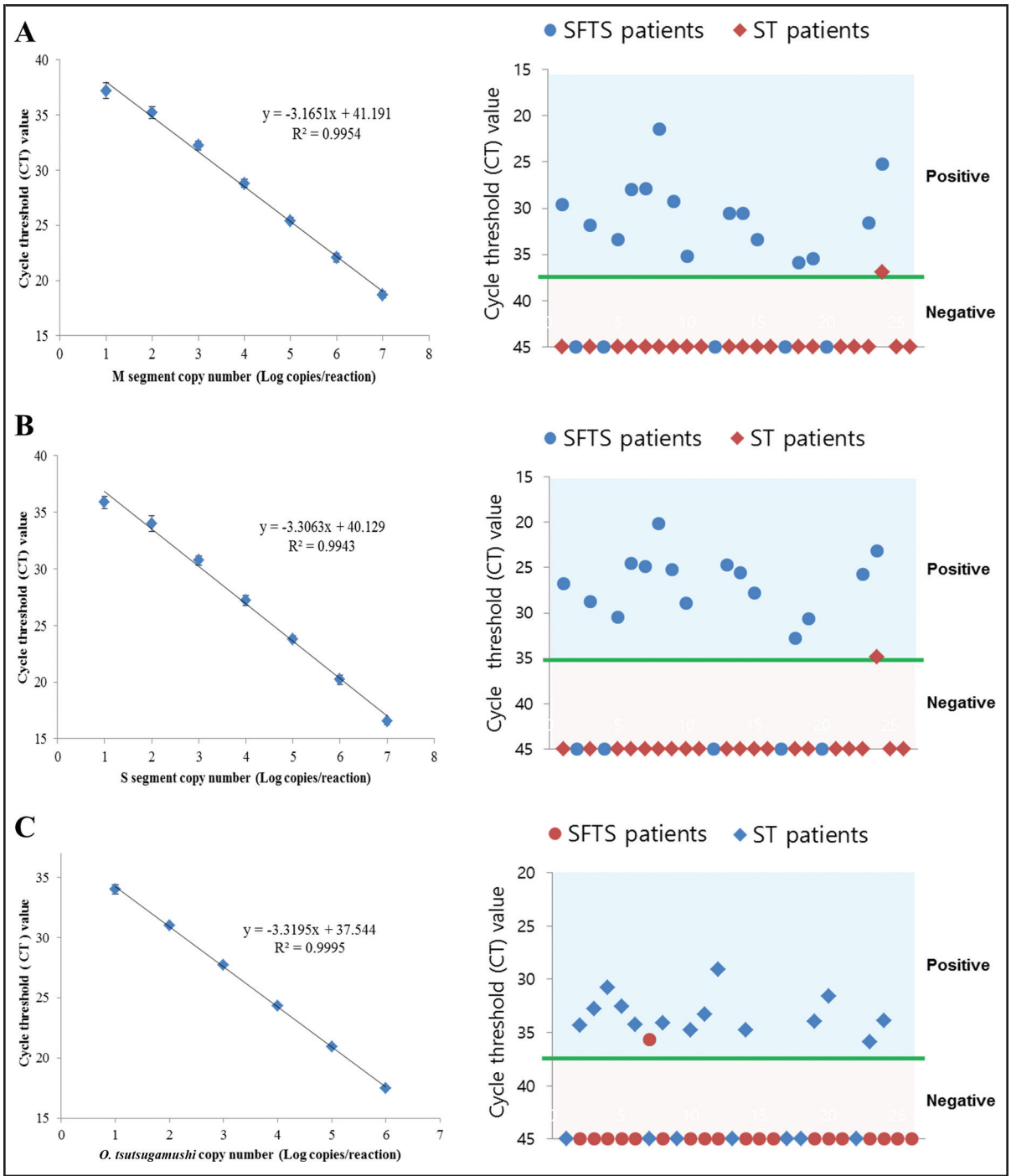

Fig. 2. Standard curves of real-time PCR and dot plots of the cycle thresholds of all serum samples.

The graphs on the left are standard curves for the SFTSV M segment (A), the SFTSV S segment (B), and scrub typhus (C) generated by linear regression plots of the cycle threshold values and in vitro synthetic SFTSV RNA or control *O. tsutsugamushi* DNA. Right figures are dot plots that represent the cycle threshold values of the serum samples. Circles represent the SFTS samples and diamonds represent the scrub typhus samples. Colors represent positive (blue) and negative (red) samples.

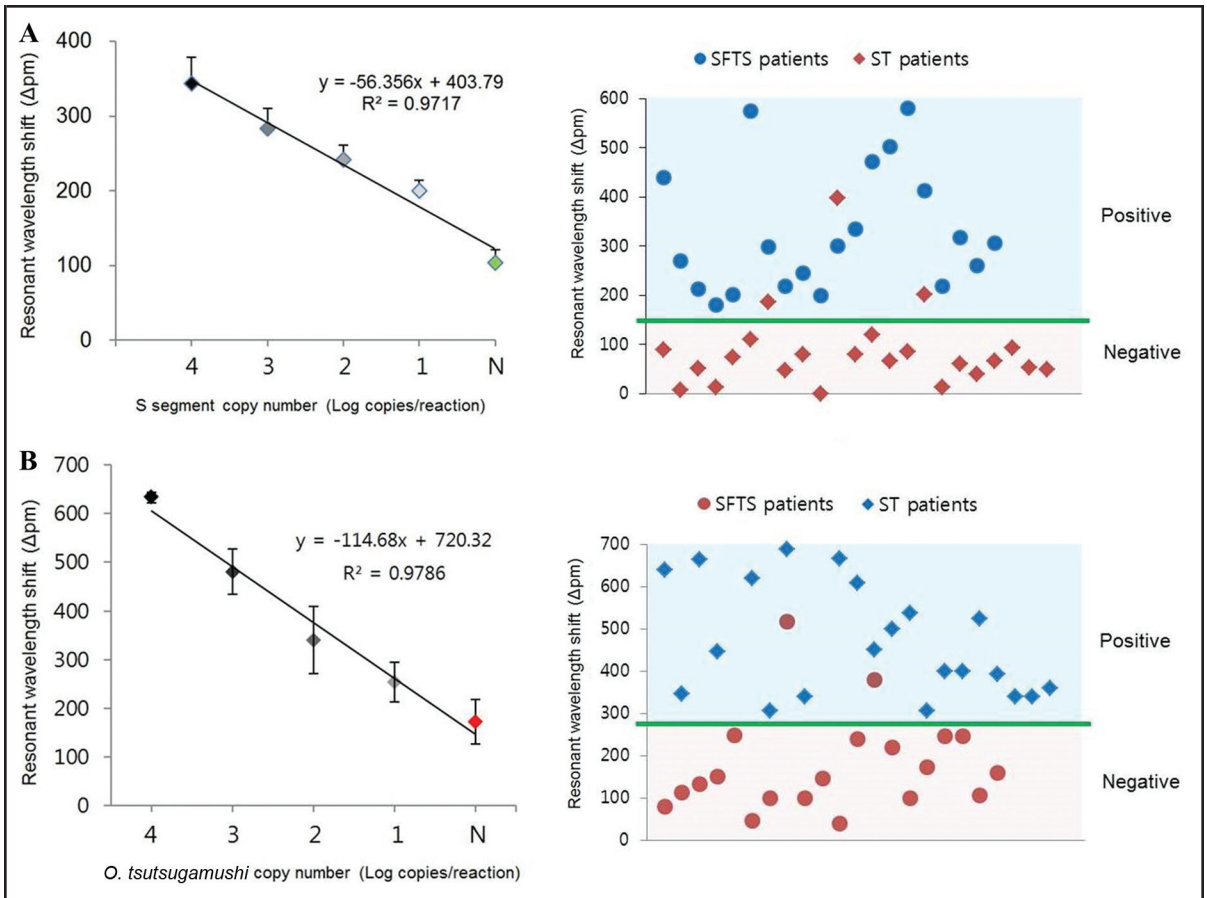

Fig. 3. Standard curves for iNAD and dot plots of resonant wavelength shifts for each serum sample.

Left figures are standard curves of SFTSV S segment (A) and *O. tsutsugamushi* (B) generated from linear regression plots of resonance wavelength shifts and in vitro synthetic SFTSV RNA or control *O. tsutsugamushi* DNA. Right figures are dot plots of the resonance wavelength shifts of each serum sample. Circles represent SFTS samples and diamonds represent scrub typhus samples. Colors represent positive (blue) and negative (red) samples of SFTS and scrub typhus.

negative results for the 5 plasma specimens from patients with late disease and iNAD gave positive results. In previous studies, the clinical diagnosis of SFTS had 94%–100% clinical sensitivity *(16, 17, 34)*, but the authors did not include serum samples from the late recovery phase. Given that conventional PCR and real-time PCR have been considered gold standards for the diagnosis of SFTS and yet are not 100% clinically sensitive, iNAD may be useful for diagnosing SFTS patients who have mild presentations, with recent or very low-level viremia.

Previous studies obtained a range of clinical sensitivities for detection of scrub typhus by real-time PCR, depending on the sample type and phase of illness. One study found that the clinical sensitivities of real-time PCR were 50% and 28.6% for whole blood and buffy coat samples, respectively, in the acute phase of scrub typhus (≤7 days of illness) *(15)*. In contrast, one group reported 65% clinical sensitivity for serum or whole blood samples *(35)*, and other studies achieved 83%–87% sensitivity for buffy coat samples *(14, 36)*. In the present study, the clinical sensitivity of real-time PCR was 67% for plasma, which is within the range of the results of previous studies. However, our data clearly showed that the clinical sensitivity of iNAD (100%) in patients with scrub typhus over the 3rd to 21st day of illness was higher than that of real-time PCR (67%; Table 3 and see Table 2 in the online Data Supplement). Furthermore, iNAD was 5 times more rapid (20 min) than real-time PCR (100 min) because in iNAD the targets are simultaneously amplified and detected without thermal cycling, in a label-free and real-time manner. Therefore, iNAD can be used to detect very low amounts of DNA in the plasma of

Table 3. The diagnostic performance of real-time PCR and iNAD with use of SFTSV- and O. tsutsugamushi-specific primers in the 15[a] patients with SFTS and 21 patients with scrub typhus.

	SFTSV-specific primer		*O. tsutsugamushi*-specific primer	
	SFTS (n = 20)	Scrub typhus (n = 21)	Scrub typhus (n = 21)	SFTS (n = 20)
Real-time PCR				
Positive (n)	15	1	14	1
Negative (n)	5[a]	20	7	19
Sensitivity (%)	75 (51–91)[b]		67 (43–85)[d]	
Specificity (%)	95 (77–99)[c]		95 (73–100)[e]	
iNAD				
Positive (n)	20	3	21	2
Negative (n)	0	18	0	18
Sensitivity (%)	100 (83–100)[b]		100 (81–100)[d]	
Specificity (%)	86 (65–97)[c]		90 (67–98)[e]	

Clinical sensitivity and specificity are presented as percentage (%) and 95% confidence interval (95% CI).
[a] Five additional plasma samples from the 15 patients with SFTS, taken on the day of the first negative results for real-time PCR for SFTS, were included in this analysis.
[b] $P = 0.047$ for the difference in sensitivity for SFTS between real-time PCR and iNAD.
[c] $P = 0.61$ for the difference in specificity for SFTS between real-time PCR and iNAD.
[d] $P = 0.009$ for the difference in sensitivity for scrub typhus between real-time PCR and iNAD.
[e] $P > 0.99$ for the difference in specificity for scrub typhus between real-time PCR and iNAD.

patients with scrub typhus and thus to rapidly rule out a diagnosis of that disease.

Although the iNAD had greater clinical sensitivities for detecting SFTS and scrub typhus, some factors still need to be improved. The specificity of iNAD appeared to be somewhat lower than that of Taqman probe-based real-time PCR, although the difference was not statistically significant. In addition, quantitative analysis by iNAD has not yet been validated. Thus, iNAD needs to be optimized on the basis of a larger cohort to improve it technically in terms of specificity and quantification of viral or bacterial loads in various clinical samples.

In conclusion, the iNAD method detects SFTSV and *O. tsutsugamushi* in plasma more rapidly and sensitively than real-time PCR. Thus, this new bio-optical sensor detection method may be useful for differentiating SFTS from scrub typhus in patients with suspected tick-borne disease.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: Grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant no. HI16C0272).
Expert Testimony: None declared.
Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or final approval of manuscript.

References

1. Seong SY, Choi MS, Kim IS. Orientia tsutsugamushi infection: overview and immune responses. Microbes Infect 2001;3:11–21.
2. Yun Y, Heo ST, Kim G, Hewson R, Kim H, Park D, et al. Phylogenetic analysis of severe fever with thrombocytopenia syndrome virus in South Korea and migratory bird routes between China, South Korea, and Japan. Am J Trop Med Hyg 2015;93:468–74.
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;364:1523–32.
4. Kim KH, Yi J, Kim G, Choi SJ, Jun KI, Kim NH, et al. Severe fever with thrombocytopenia syndrome, South Korea, 2012. Emerg Infect Dis 2013;19:1892–94.
5. Takahashi T, Maeda K, Suzuki T, Ishido A, Shigeoka T, Tominaga T, et al. The first identification and retrospective study of Severe Fever with Thrombocytopenia Syndrome in Japan. J Infect Dis 2014;209:816–27.
6. Liu S, Chai C, Wang C, Am S, Lv H, He H, et al. Systematic review of severe fever with thrombocytopenia syndrome: virology, epidemiology, and clinical characteristics. Rev Med Virol 2014;27:90–102.
7. Korea Centers for Disease Control and Prevention (KCDC). Disease information, SFTS.http://cdc.go.kr/CDC/health/CdcKrHealth0101.jsp?menuIds=HOME001-MNU1132-MNU1147-MNU0746-MNU2423&fid=7956&cid=70361 (Accessed November 2016).
8. Tamura A, Ohashi N, Urakami H, Miyamura S. Classification of Rickettsia tsutsugamushi in a new genus, Orien-

tia gen. nov., as Orientia tsutsugamushi comb. nov. Int J Syst Bacteriol 1995;45:589–91.
9. Watt G, Parola P. Scrub typhus and tropical rickettsioses. Curr Opin Infect Dis 2003;16:429–36.
10. Kim WY, Choi W, Park SW, Wang EB, Lee WJ, Jee Y, et al. Nosocomial transmission of severe fever with thrombocytopenia syndrome in Korea. Clin Infect Dis 2015;60:1681–83.
11. 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 2001;44:391–400.
12. La Scola B, Raoult D. Laboratory diagnosis of rickettsioses: current approaches to diagnosis of old and new rickettsial diseases. J Clin Microbiol 1997;35:2715–27.
13. Paris DH, Aukkanit N, Jenjaroen K, Blacksell SD, Day NP. A highly sensitive quantitative real-time PCR assay based on the groEL gene of contemporary Thai strains of *Orientia tsutsugamushi*. Clin Microbiol Infect 2009;15:488–95.
14. 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;55:1377–87.
15. 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;98:308–10.
16. 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.
17. Li Z, Qi X, Zhou M, Bao C, Hu J, Wu B, et al. A two-tube multiplex real-time RT-PCR assay for the detection of four hemorrhagic fever viruses: severe fever with thrombocytopenia syndrome virus, Hantaan virus, Seoul virus, and dengue virus. Arch Virol 2013;158:1857–63.
18. Shin Y, Perera AP, Kim KW, Park MK. Real-time, label-free isothermal solid-phase amplification/detection (ISAD) device for rapid detection of genetic alteration in cancers. Lab Chip 2013;13:2106–14.
19. Koo B, Jin CE, Lee TY, Lee JH, Park MK, Sung H, et al. An isothermal, label-free, and rapid one-step RNA amplification/detection assay for diagnosis of respiratory viral infections. Biosens Bioelectron 2017;90:187–94.
20. Kim DM, Lee YM, Back JH, Yang TY, Lee JH, Song HJ, et al. A serosurvey of *Orientia tsutsugamushi* from patients with scrub typhus. Clin Microbiol Infect 2010;16:447–51.
21. Seong SY, Huh MS, Jang WJ, Park SG, Kim JG, Woo SG, et al. Induction of homologous immune response to *Rickettsia tsutsugamushi* Boryong with a partial 56-kilodalton recombinant antigen fused with the maltose-binding protein MBP-Bor 56. Infect Immun 1997;65:1541–1545.
22. Blacksell SD, Luksameetanasan R, Kalambaheti T, Aukkanit N, Paris DH, McGready R, et al. Genetic typing of the 56-kDa type-specific antigen gene of contemporary Orientia tsutsugamushi isolates causing human scrub typhus at two sites in north-eastern and western Thailand. FEMS Immunol Med Microbiol 2008;52:335–42.
23. Bakshi D, Singhal P, Mahajan SK, Subramaniam P, Tuteja U, Batra HV. Development of a real-time PCR assay for the diagnosis of scrub typhus cases in India and evidence of the prevalence of new genotype of *O. tsutsugamushi*. Acta Trop 2007;104:63–71.
24. Furuya Y, Yoshida Y, Katayama T, Yamamoto S, Kawamura A. Serotype-specific amplification of *Rickettsia tsutsugamushi* DNA by nested polymerase chain reaction. J Clin Microbiol 1993;31:1637–40.
25. Kyoto University Bioinformatics Center. Multiple sequence alignment by Clustal W. http://www.genome.jp/tools/clustalw/ (Accessed October 2017).
26. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. Primer3–new capabilities and interfaces. Nucleic Acids Res 2012;40:e115.
27. Iqbal M, Gleeson MA, Spaugh B, Tybor F, Gunn WG, Hochberg M, et al. Label-free biosensor arrays based on silicon ring resonators and high-speed optical scanning instrumentation. IEEE J Sel Top Quantum Electron 2010;16:6654–61.
28. Bogaerts W, De Heyn P, Van Vaerenbergh T, De Vos K, Kumar Selvaraja S, Claes T, et al. Silicon microring resonators. Laser Photonics Rev 2012;6:47–73.
29. Piepenburg O, Williams CH, Stemple DL, Armes. NA DNA detection using recombination proteins. PLoS Biol 2006;4:e204.
30. Choi SY, Park SW, Bae IG, Kim SH, Ryu SY, Kim HA, et al; for Korea SFTS Clinical Network. Severe fever with thrombocytopenia syndrome in South Korea, 2013–2015. PLoS Negl Trop Dis 2016;10:e0005264.
31. Jeung YS, Kim CM, Yun NR, Kim SW, Han MA, Kim DM. Effect of latitude and seasonal variation on scrub typhus, South Korea, 2001–2013. Am J Trop Med Hyg 2016;94:22–5.
32. 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;52:3325–33.
33. Zhou X, Zhang T, Song D, Huang T, Peng Q, Chen Y, et al. Comparison and evaluation of conventional RT-PCR, SYBR Green I and TaqMan real-time RT-PCR assays for the detection of porcine epidemic diarrhea virus. Mol Cell Probes 2017;33:36–41.
34. Cui L, Ge Y, Qi X, Xu G, Li H, Zhao K, et al. Detection of severe fever with thrombocytopenia syndrome virus by reverse transcription-cross-priming amplification coupled with vertical flow visualization. J Clin Microbiol 2012;50:3881–5.
35. Kramme S, An V, Khoa ND, Trin V, Tannich E, Rybniker J, et al. Panning, Orientia tsutsugamushi bacteremia and cytokine levels in Vietnamese scrub typhus patients. J Clin Microbiol 2009;47:586–9.
36. Kim DM, Park G, Kim HS, Lee JY, Neupane GP, Graves S, Stenos J. Comparison of conventional, nested, and real-time quantitative PCR for diagnosis of scrub typhus. J Clin Microbiol 2011;49:607–12.