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Detection of Severe Fever with Thrombocytopenia Syndrome Virus by Reverse Transcription–Cross-Priming Amplification Coupled with Vertical Flow Visualization

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A virus known as severe fever with thrombocytopenia syndrome virus (SFTSV) was recently identified as the etiological agent of severe fever with thrombocytopenia syndrome (SFTS) in China. Reliable laboratory detection and identification of this virus are likely to become clinically and epidemiologically desirable. We developed a nearly instrument-free, simple molecular method which incorporates reverse transcription–cross-priming amplification (RT-CPA) coupled with a vertical flow (VF) visualization strip for rapid detection of SFTSV. The RT-CPA-VF assay targets a conserved region of the M segment of the SFTSV genome and has a limit of detection of 100 copies per reaction, with no cross-reaction with other vector-borne bunyaviruses and bacterial pathogens. The performance of the RT-CPA-VF assay was determined with 175 human plasma specimens collected from clinically suspected SFTS patients and 86 healthy donors. The sensitivity and specificity of the assay were 94.1% and 100.0%, respectively, compared with a combination of virus culture and real-time RT-PCR. The entire procedure, from specimen processing to result reporting, can be completed within 2 h. The simplicity and nearly instrument-free platform of the RT-CPA-VF assay make it practical for point-of-care testing.

In 2009, a novel virus within the family Bunyaviridae was identified as the etiologic agent of severe fever with thrombocytopenia syndrome (SFTS) in China (16). The virus, subsequently named severe fever with thrombocytopenia syndrome bunyavirus (SFTSV), is associated with a clinical syndrome characterized by fever, respiratory or gastrointestinal symptoms, thrombocytopenia, leukocytopenia, and hemorrhage in severe cases. SFTS has a mortality rate of around 12% and is believed to have existed for some time and to have a wide geographic distribution (9, 13, 16). The Haemaphysalis longicornis tick was found to carry SFTSV, suggesting that it is a possible vector of SFTSV infection to humans. Recently, cases of person-to-person transmission have been reported (1, 5, 10). Clinical features of SFTS are nonspecific and overlap those of human granulocytic anaplasmosis (HGA; caused by Anaplasma phagocytophilum), hemorrhagic fever with renal syndrome, and leptospirosis (16). Therefore, a reliable SFTSV detection tool is urgently needed to provide early diagnosis of SFTS to facilitate clinical care, infection control, and epidemiologic investigations. Currently, one quantitative real-time reverse transcription-PCR (RT-PCR) assay and one double-antigen sandwich enzyme-linked immunosorbent assay (ELISA) have been developed (7, 12).

Cross-priming amplification (CPA) (Chinese IP Office patent application 200810134583.1, application date 29 July 2008, U.S. provisional patent 6965028, and PCT patent PCT/US2010/000024, publication date 15 July 2010) is an isothermal DNA amplification method developed by Ustar Biotechnologies (Hangzhou, China). CPA has demonstrated high specificity and sensitivity, producing amplicons from as few as four bacterial cells (14). A vertical flow (VF) hybridization-based nucleic acid detection strip cassette was designed to visually detect double-labeled amplicons in 5 to 10 min (8). CPA coupled with a VF visualization strip has been developed successfully to detect specific DNAs from several bacterial pathogens, including Mycobacterium tuberculosis and Enterobacter sakazakii (3, 17). In this study, CPA was modified to include reverse transcription-CPA (RT-CPA) technology to rapidly detect SFTSV-specific RNA. The performance of the RT-CPA-VF assay in detecting SFTSV directly from clinical specimens was also evaluated.

MATERIALS AND METHODS

Viral isolates and clinical specimens. Ten SFTSV strains isolated from patient samples at the Jiangsu Provincial Center for Disease Prevention and Control (JS4, JS6, JS14, JS15, JS18, JS19, JS24, JS26, and JS2007-01) and one strain isolated from a dog (JSD1) were included in the RT-CPA-VF assay development. The dog isolate (JSD1) has >95.2% nucleotide sequence homology with the isolates from patients. Eighty-nine serum specimens were collected from suspected SFTS patients in the acute phase of illness, during 2010 and 2011. Among them, 51 were confirmed to have SFTSV infection by virus isolation (n = 26), a real-time RT-PCR assay (n = 51), or both (n = 26). Eighty-six serum specimens from healthy subjects who were receiving routine care in areas where the disease is not
endemic were also included in the study. Genetically and clinically related bacterial and viral pathogens used in this study included Hantavirus virus (strain 76-118), Seoul virus (strain R22), dengue fever virus (strain JS01/10), Anaplasma phagocytophilum, Leptospira interrogans (Jiangsu Provincial Center for Disease Prevention and Control), Rift Valley fever virus (strain ZH501), Crimean-Congo hemorrhagic fever virus (strain IBAr10200), Russian spring-summer encephalitis virus (strain Sofjin) (University of Texas Medical Branch, Galveston, TX), Ehrlichia ewingii, and Ehrlichia chaffensis (Vanderbilt University Medical Center). This project was approved by the Ethics Committee of the Jiangsu Provincial Center for Disease Prevention and Control, and written informed consent was obtained from participants.

Recombinant transcript RNA. To amplify a fragment carrying the T7 promoter, RT-PCR was carried out with SFTSV strain JS26, using primers containing the T7 promoter sequence in the reverse direction. RNA was transcribed directly from the purified PCR product by use of T7 RNA polymerase (TaKaRa, Dalian, China) according to the manufacturer’s instructions. The amount of transcript was measured by spectrophotometry at 260 nm. The quantity (copies/µl) of RNA standard was calculated according to a previously described formula (4). Aliquots of the dilution series were frozen at −80°C until used.

Viral culture. Two-hundred-microliter samples of real-time RT-PCR (see below)-positive serum specimens were inoculated onto Vero cell monolayers. Cells were monitored daily for the presence of cytopathic effect (CPE) or viral RNA in the supernatant by real-time PCR. Once suspected CPE occurred, an indirect immunofluorescence assay was performed to determine the specificity for SFTSV. The cells were cultured at 37°C in a 5% carbon dioxide atmosphere, with medium changes twice a week.

RNA extraction. RNAs were extracted from 200-µl samples of culture supernatants of 11 SFTS virus strains or patient serum specimens by use of a High Pure viral RNA kit (Roche Diagnostics, Manheim, Germany) following the manufacturer’s instructions. The purified RNA was distilled in 50 µl diethyl pyrocarbonate-treated water and stored at −80°C until further testing.

Primer design. SFTSV-specific primers for RT-CPA were designed based on the sequence of the M segment. The complete sequence of the SFTSV M segment, available in the GenBank database, was evaluated using DNAStar software to identify a conserved region. A set of five primers comprising two displacement primers, one cross primer, and two detector primers was designed with Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA). The two displacement primers were designated “displacement primer sense” (4s) and “displacement primer antisense” (5a). The detector primers were designated “detector primer 1 sense” (2s), labeled with biotin at the 5’ end, and “detector primer 2 sense” (3s), labeled with fluorescein isothiocyanate (FITC) at the 5’ end. The cross primer was composed of the detector primer 1 sense (2s) sequence at the 5’ end and 1a sequence at the 3’ end. The locations and sequences of the oligonucleotide primers are shown in Fig. 1. All of the primers used were purchased from Sangon (Shanghai, China).

RT-CPA-VF assay. RT-CPA reactions were carried out in 20-µl reaction mixtures containing 0.3 mM (each) primers 3a and 2a, 0.5 mM cross primer 1s, 0.3 mM (each) displacement primers 4s and 5a, a 0.8 mM concentration of each deoxyribonucleoside triphosphate (dNTP), 1 mM MgSO4, 0.1% Triton X-100, 8 U Br DNA polymerase large fragment (New England BioLabs), 10 U of the avian myeloblastosis virus reverse transcriptase (Invitrogen), and 2 µl of target RNA. The CPA reaction was carried out at 60°C for 1.5 h in a heating block, followed by heating at 80°C for 2 min to terminate the reaction. The reaction tube was then placed directly into the VF visualization strip cassette (Ustar Biotech Co., Ltd., Hangzhou, China) for amplification detection (Fig. 2A).

Real-time RT-PCR. The real-time RT-PCR assay for SFTSV was adapted from the work of Sun et al. (12), with modifications. Previous full validation revealed that the assay had a sensitivity of 98.6% and a specificity of 99.0% (12). In brief, primers (5’-GGG TCC CTG AAG GAG TTG TAA A-3’ and 5’-TGC CTT CAC CAA GAC GAT TAA CTT GCT GGC TCC GCG C-BHQ-3’) were designed to target the conserved nucleic acid sequences in the 5’ end. Real-time RT-PCR variants were performed using an AgPath-ID one-step RT-PCR kit (Applied Biosystems), and reaction mixtures contained 10 µl of 2× RT-PCR buffer, 400 nM primer mix, 100 nM probe mix, 0.8 µl of RT-PCR enzyme mix, and 4 µl of total RNA extract as the template, in a final volume of 20 µl. Real-time PCR cycling was performed on an ABI 7500 system as follows: after reverse transcription at 50°C for 30 min, the Taq polymerase was activated at 95°C for 10 min, and amplification was undertaken for 40 cycles, consisting of a denaturation step at 95°C for 15 s and an annealing-extension step at 60°C for 45 s.

Reference standard. The reference standard represented the combined results of viral culture and real-time RT-PCR. A sample was determined to be positive for SFTSV when viral culture or real-time RT-PCR was positive.

RESULTS
Development of the RT-CPA-VF assay. A representative schematic and the result readout of RT-CPA are shown in Fig. 2 and 3. A positive result was shown as two visible red lines, at the test and control line positions, while a negative reaction demonstrated a red line only at the control line, within 5 to 10 min (Fig. 2B).

Limit of detection and analytical specificity of RT-CPA-VF assay. The limit of detection (LOD) of the RT-CPA-VF assay for SFTSV was determined by testing serial 10-fold dilutions of in vitro-transcribed standard RNAs ranging from 0 to 100,000 copies/reaction mix. Each dilution was performed in triplicate. The RT-CPA assay detected all reaction products with more than 10 copies/reaction mix. Among the dilutions of 0, 1, and 10 copies/reaction mix, the RT-CPA assay detected only one of three 10-copy/reaction tubes. The results indicate that the RT-CPA-VF assay has an LOD of 102 copies of standard transcript per reaction mix.

The analytical specificity of the RT-CPA-VF assay was evaluated by RT-CPA-VF amplification of viral RNA extracts from 11 SFTSV strains and other related pathogens (roughly 10,000 copies of RNA for each species). The test was positive only for the 11 SFTSV strains and was negative for Hantaan virus, Seoul virus,
Rift Valley fever virus, Crimean-Congo hemorrhagic fever virus, Russian spring-summer encephalitis virus, dengue fever virus, Leptospira interrogans, Anaplasma phagocytophilum, Ehrlichia ewingii, and E. chaffeensis. The results indicate that the RT-CPA-VF assay has a 100% analytical specificity for SFTSV detection.

Clinical performance. Of 89 serum samples from suspected SFTS patients, 48 tested positive by both the reference method and the RT-CPA-VF assay. Three reference-positive specimens tested negative by the RT-CPA-VF assay, resulting in a sensitivity of 94.1% (Table 1). SFTSV was not detected by RT-CPA-VF assay in all 86 specimens from healthy subjects, resulting in a specificity of 100% (Table 1).

DISCUSSION

In this study, we developed an RT-CPA-VF assay for rapid detection of SFTSV as a potential point-of-care test (POCT). Because the viral loads in the sera of individuals infected with SFTSV can be as high as $8.2 \times 10^7$ copies per milliliter and viral RNA levels gradually decline over 3 to 4 weeks after the onset of illness (18), detection methods targeting SFTSV-specific nucleic acid sequences are a logical choice for early diagnosis in clinical settings. Almost all reported SFTS patients were farmers or residents who lived in mountainous or hilly areas, and peripheral health care settings were usually their first consultation sites (13, 16). Thus, a detection method that can be used in primary care facilities and smaller clinical laboratories is necessary. A rapid and simple detection method that can be used as a POCT in a remote area is ideal for SFTS clinical patient care and epidemiological investigation. The RT-CPA-VF assay developed in the present study fulfills all these characteristics.

Several other isothermal amplification methods have been developed that offer simplified formats that could pave the way for molecular diagnosis of infectious pathogens. These include strand displacement amplification (SDA), transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), loop-mediated amplification (LAMP), rolling-circle amplification (RCA), and helicase-dependent amplification (HDA). SDA and TMA have been developed into high-throughput devices, mainly for the U.S. and European markets. Additional fluorescence or electrogenerated chemiluminescence detection instruments are needed for NASBA assays. LAMP is a new and promising low-cost method that has been used to detect various infectious pathogens. A major limitation of LAMP assays is the requirement for primers with high stringency, which requires special software programs (11).

Like other isothermal amplification techniques, the CPA reaction allows for target sequence amplification at a constant temperature. The rationale and mechanism of CPA were recently described by Xu et al. (14). The only instrument required for amplification is a heat block or water bath. Multiple cross-linked primers (five to eight primers) are used in a CPA reaction mix and enormously increase the amplification efficiency (14). The entire process of amplification and detection can be finished within 2 h, similar to the time required for conventional real-time RT-PCR. In the CPA assay, two primers involved in isothermal amplification are labeled, one with biotin and one with FITC. During the reaction stage, the amplified products are labeled simultaneously. The end of the amplicon labeled with biotin binds streptavidin-conjugated color particles for visualization. The other end, labeled with FITC, is captured by an anti-FITC antibody located on the test line of the strip, and the test results are shown as a colored line visible by the naked eye in 5 to 10 min (2, 6, 8, 15). Importantly, the VF strip is housed in an enclosed, sealed plastic device to prevent the leakage of amplicons which may cause cross-contamination and false-positive results. Isothermal amplification coupled
with a VF visualization strip provides a simple, cost-effective, and easily interpretable format for molecular testing (8).

In the present study, the RT-CPA-VF assay was established using a previously described CPA technique, but with the addition of reverse transcriptase and optimized pH and ion concentrations. This study demonstrates for the first time that RNA can be used successfully as an amplification template for CPA. A 125-nucleotide fragment of the M segment of SFTSV was selected for primer design, since the sequences of this segment showed much more variance than that for other viruses in the genus Phlebovirus (4).

To test the specificity of the primers, 11 strains of SFTSV and 10 strains of other microbes that are closely related by phylogenetic analysis or cause similar clinical syndromes were selected. The RT-CPA-VF assay showed 100% specificity for the selected panel. Although the sensitivity of the RT-CPA-VF assay was slightly lower than that of the previously described real-time RT-PCR assay (6), clinical specimens tested by the RT-CPA assay demonstrated a 94.1% agreement between the two methods. The results showed a high sensitivity and specificity of the RT-CPA-VF assay for detection of SFTSV infection.

### TABLE 1 Performance of RT-CPA method in detecting clinical samples

<table>
<thead>
<tr>
<th>RT-CPA result</th>
<th>No. of samples with reference method result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>48 Positive, 0 Negative</td>
</tr>
<tr>
<td>Negative</td>
<td>38 Positive by real-time RT-PCR but negative by culture</td>
</tr>
</tbody>
</table>

*The sensitivity and specificity of the RT-CPA method were 94.1% and 100%, respectively.*

**Fig 3** Schematic diagram of the mechanisms for RT-CPA. RNA was reverse transcribed into cDNA by reverse transcriptase using displacement primer 4s (step 1). After cross primer 1a anneals and extends, displacement primer 5a anneals and extends, displacing the downstream strand. The displaced strand had a new primer binding site (2s sequence) added at the 5’ end (step 2). This strand was used as a template by primers 2s and 3s and displacement primer 4s, which anneal and extend in tandem, creating two different single-stranded DNA products (step 3). The left, FITC-labeled 3s’ product was extended by the biotin-labeled 2s primer to form a double-labeled detectable product (step 4). The right, biotin-labeled 2s product formed double-stranded DNA by cross primer 1a extension (step 4). The sense strand of the resulting double-stranded DNA acted as the template for further extension by primer 1a (step 5). The antisense strand acted as the template for further extension by primers 2s and 3s, forming a FITC-labeled 3s’ product and a biotin-labeled 2s product (step 5). The biotin-labeled 2s product was the same as the sense strand of the resulting double-stranded DNA, while the FITC-labeled 3s product was extended by the biotin-labeled 2s primer to further form a double-labeled detectable product.
In conclusion, our study demonstrates that the RT-CPA-VF method provides an attractive laboratory tool for the detection of SFTSV in clinical specimens. This rapid detection method does not require expensive equipment. In addition, the sealing device used for CPA detection avoids cross-contamination between specimens, and the results are easy to interpret. The high sensitivity and specificity of the RT-CPA-VF method for the identification of SFTSV and its ease of use and low cost make the RT-CPA-VF assay an ideal method for use in primary care facilities and clinical laboratories in resource-limited settings.

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