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A RISPR-based nucleic acid detection method for severe fever with thrombocytopenia syndrome virus

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ABSTRACT

Objective: Fever with thrombocytopenia syndrome virus (SFTS) is a tick-borne infection now known to spread among humans as an aerosol, which has resulted in several outbreaks across Asia over the past decade. As mortality is substantial, it is vital to establish a rapid, on-site nucleic acid detection method for diagnosis. Here we describe such a method for SFTSV (*Dabie bandavirus*) based on CRISPR-Cas13a.

Methods: Specific recombinase-aided amplification (RAA) primers and CRISPR (cr)RNA nucleic acid detection targets were designed and synthesized for the conserved sequence of the SFTSV genome, and fluorescent CRISPR detection was used to screen for high-sensitivity crRNAs. Colloidal immunochromatography test paper was used to read CRISPR detection results. Sensitivity and specificity were evaluated by running tests on gradient dilutions of SFTSV nucleic acid and the nucleic acids of other pathogens with similar transmission routes or clinical manifestations.

Results: One crRNA with high detection sensitivity was screened out of 5 crRNAs with conserved sequences from the SFTSV genome. This CRISPR nucleic acid-based detection method was able to detect a single crRNA copy per microliter but not the nucleic acids of similar pathogens.

Conclusion: This CRISPR test strip detection method permits rapid, sensitive, and specific diagnosis of SFTS without the need for advanced nucleic acid detection equipment, thus allowing for on-site application.

1. Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is a recently identified zoonotic (tick-borne) illness caused by the Bunia virus *Dabie bandavirus*. The causative pathogen is a single- negative-stranded enveloped RNA virus (Lei et al., 2014) that contains three gene segments: large (L), medium (M), and small (S). As an emerging infectious disease, the incubation period of the disease is unknown, although the onset of symptoms is usually rapid. It was first discovered in Anhui Province, China, and confirmed cases were subsequently found in more than ten provinces and cities in China as well as South Korea, Japan, and the United States among other countries (Wu and Zhang, 2017). The main clinical manifestations are fever and thrombocytopenia (Li, 2011). Further, SFTSV is highly transmissible and mortality rates up to 30% have been reported, mainly due to multiple organ failure (Li, 2011), so SFTS is a serious emerging public health concern.

Research on SFTSV is still in its infancy, and there are currently no effective vaccines and drugs for prevention and treatment. Therefore, effective control of disease spread requires rapid and accurate on-site testing for diagnosis. Virus isolation and sequencing is the gold standard for diagnosis of infection, but is time consuming and the requisite equipment may be unavailable at the epidemic site. Enzyme-linked immunosorbent assay (ELISA) detection technology is relatively mature, but accuracy is influenced by many factors, especially temperature and incubation time, and is hampered to serological cross-reaction (Ishino et al., 1987). Alternatively, nucleic acid detection and identification using RT-PCR is fast and accurate, but is also expensive and difficult to apply on-site.

In recent years, the clustered regularly interspaced short palindromic repeat (CRISPR) system, a defense system evolved in bacteria to protect

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against viral infection, has emerged as a powerful tool for gene editing and the detection of pathogenic microorganisms. The CRISPR system includes the DNA-targeting CRISPR-related nucleases Cas9, Cas12 and RNA-targeting CRISPR-related nucleases Cas13, all of which have been used in pathogen detection. When Cas13a binds a crRNA and a ssRNA, the indiscriminate cleavage activity of Cas13a will be activated. In that state, Cas13a is able to cleave the target ssRNA as well as adjacent reporter ssRNA, termed "collateral cleavage", that in turn allow sensitive detection of single-molecule RNA (Abudayyeh et al., 2016; Pang and Li, 2013b).

Gotenberg (Kersting et al., 2014) and others have developed an *in vitro* nucleic acid sequence detection platform called Sherlock by combining constant temperature nucleic acid amplification (termed recombinase-mediated isothermal nucleic acid amplification technology, RAA) and CRISPR technology. The RAA system can successfully amplify the target sequence, even if at trace amounts, within about 30 min at 37–42 °C (Gootenberg et al., 2017). The amplified DNA sequence is then transcribed *in vitro* by T7 RNA polymerase, and the generated RNA molecule is detected using a reporter RNA guided by Cas13 (Fig. 1). This technology has shown good sensitivity and specificity for the detection of Zika virus, dengue virus (Pardee et al., 2016), and nucleic acid sequences from other pathogens. In the present study, we combined CRISPR-Cas13a nucleic acid detection technology and RAA to establish a simple, rapid, highly sensitive, and highly specific nucleic acid detection technology for SFTSV, providing a new method for on-site diagnosis.

2. Materials and methods

2.1. Materials

2.1.1. Gene sequence and standard materials

The complete sequences of the SFTSV genome L segment (6368 bp), M segment (3378 bp), and S segment (1744 bp) were downloaded from Genbank (www.ncbi.nlm.nih.gov/genbank/), and subsequent bioinformatic analyses were conducted using MEGA X (www.megasoftware.net/show_eua) and NCBI-BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 1). A viral reference product including six SFTSV strains (AH12, HN1, JS3, LN3, SD4, HB29) and five negative reference strains (Japanese encephalitis virus vaccine strain SA14-14-2, dengue virus type 4 strain Ban18, yellow fever virus strain 17D, HFRS VIRUS strain L99, and forest encephalitis virus strain Senzhang) was obtained from the China Institute for Food and Drug Control (Lu et al., 2017). Pathogen plasmids and related primers were synthesized by Beijing Tianyi Huiyuan Biotechnology (China), and included nucleic acids from hantavirus, influenza A virus (H1N1), Japanese encephalitis virus (JEV), dengue fever virus (DENV), and tick-borne forest encephalitis virus (TBEV) among others.

2.1.2. Reagents and instruments

Main reagents included a T7 transcription kit (New england biolabs, E2050S)

SanPrep Column Plasmid DNA Mini Extraction Kit (Sangon Biotech, B518191-0050), RT-RAA amplification kit (Zhongce Biolabs, S003ZC), RNA purification magnetic beads (Beckman, A63987), a nucleic acid extraction kit (Qiagen, 52,904), Cas13 nuclease (Genscript, Z03486), Cas12/13 special nucleic acid detection test strips (Warbio, JY0301), and an RT-PCR kit (Tiangen, KR113).

Main instruments included a T100PCR thermocycler (Bio-Rad)

ThermoMixer C constant temperature metal bath (Eppendorf), fluorescence quantitative PCR instrument (QuantStudio3), desktop refrigerated centrifuge (5417R, Eppendorf), THZ-98AB constant temperature oscillator (Bluepard), electronic balance (Anyload, ES-103HA), microwave oven (Aeco, M1-L213C), and a DYY-6C electrophoresis power supply (CGOLDENWALL).

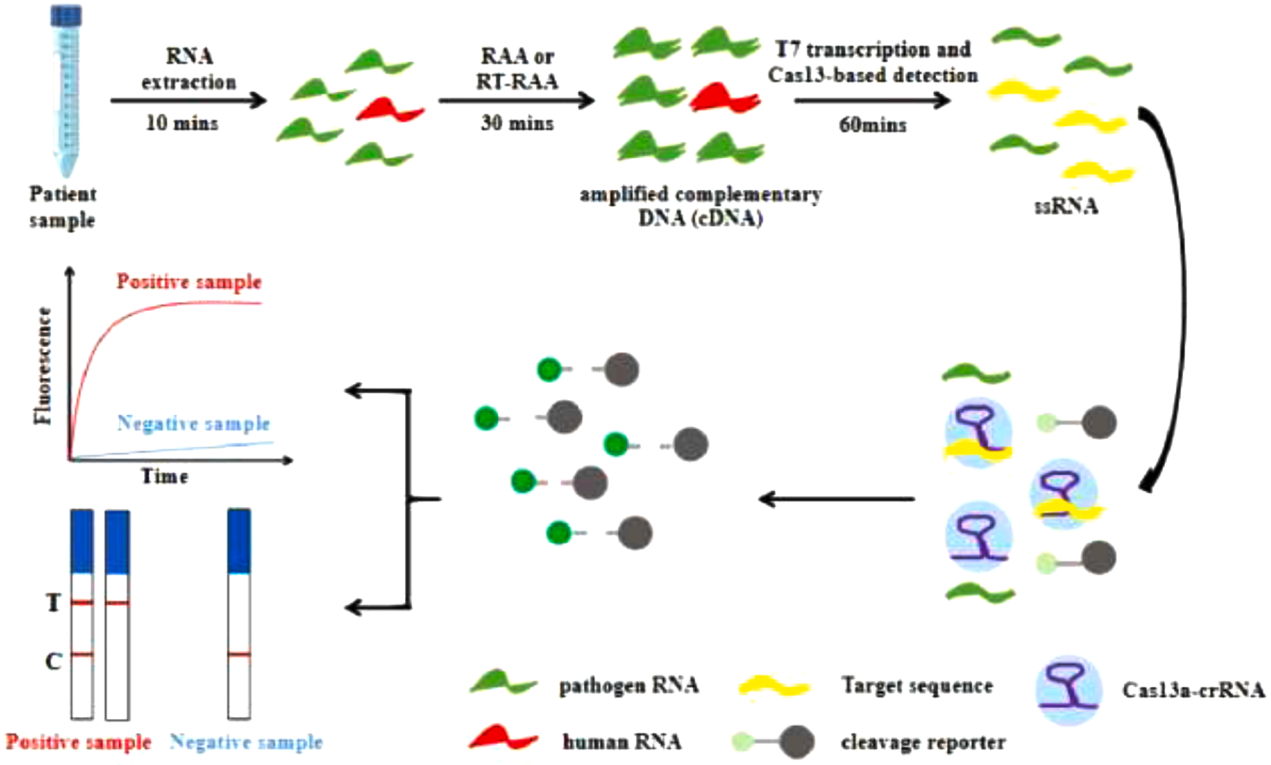


Fig. 1. SHERLOCK system combining RAA pre-amplification, Cas13 detection, and colorimetric reporter for visualized readout.

Table 1
Nucleic acid sequences used for assay development and testing.

Name	Sequence
SFTSV GenBank ID:HQ141612.1	AGCTCTCTTGCGAAA
	TCCTCAAGCTCAGTCAAATTGAGCTGCTGCTCACCAAATTCCACTG
	CAATCCTGGACCACTCTGACATGATCACTCCTTTGCGTCTTTCCTTTTTTGGGGGTCTTTGTGT
Hantavirus. GenBank ID:AF336826.1	TCCAGACTCATTATTCTCATTCAGA
	CATACTATGGTGCTGTTACGAAGGTTATTTCCTCAGGATTCCATTTCCTCTTTCTATTAGGCTTTCTTTCTTTTTCATTTTCCGGAGCGT
	ACTACTACTA
H1NI GenBank ID:KF897799.1	CTACAGGACCTGCAAGTTAGTGG
	GAATCAACATGAGCAAAAAGAAGTCCTATATAAA
	TAAGACAGGGACATT
	TGAATTCACAAGCTTTTTTTATCGCTATG
JEV GenBank ID:JX131374.1	CTCTGGATG
	GGCGTCAACGCACGAGACCGATC
	AATTGCTTTGG
	CCTTCTTAGCCACAGGAGGTGTGCTCGTGTTCTTA
	GCGACCAATGTGCATGCTGACA
	CTGGATGTGCCATTGACATCACAAGAAAAGAGATGAG
DENV GenBank ID:JQ287662.1	AGATATCATTGGCCAGAGGATAGAGAACATAAAACATGAACACAAGTCAACATGGCATTATGATGAGGACAATCCATATAAAACATGGGCCTATCATGGATCATATGAGGTCAAGCCATCAGGATCAGCCT
TBEV GenBank ID:MN114637.1	AGTCGTGAACGTGTTGAGAA
	AAAGACAGCTTAGGAGAACAAGAGCTGGGAATGGCCAGGAAGGCCATTCTGAAAGGAAAGGGGGGCG
	GTCCCCCTCGACGAGTGTCGAAAGAGACCGCGAAGAAGACGCGTCAATCCAGAGTCCAAATGCCAAATGGACTCGTGTTGATGCGCATGATGGGGATCTTGTGGCATGCC
T7-crRNA-F	TAATACGACTCACTATAGGGGATTTAGACTACCCCAA
crRNA1	TCACCAAATTCCACTGCAAT
	GGGATTTAGACTACCCCAAAAACGAAGGGGACTAAAACGGTCCAGGATTGCAGTGGAATTTGGTGA
crRNA2	GCTGCTCACCAAATTCCACT GGGATTTAGACTACCCCAAAAACGAAGGGGACTAAAACAGGATTGCAGTGGAATTTGGTGAGCAGC
crRNA3	AAATTCCACTGCAATCCTGG
	GGGATTTAGACTACCCCAAAAACGAAGGGGACTAAAACAGAGTGGTCCAGGATTGCAGTGGAATTT
crRNA4	GAGCTGCTGCTCACCAAATT GGGATTTAGACTACCCCAAAAACGAAGGGGACTAAAACTGCAGTGGAATTTGGTGAGCAGCAGCTC
crRNA5	CCACTGCAATCCTGGACCAC GGGATTTAGACTACCCCAAAAACGAAGGGGACTAAAACATGTCAGAGTGGTCCAGGATTGCAGTGG
RAA1	aattctaatacgactcactatagggGCGAAATCCTCAAGCTCAGTCAAATT AAAAAGGAAAGACGCAAAGGAGTGATC
RAA2	aattctaatacgactcactatagggGCTCTCTTGCGAAATCCTCAAGCTC ACGCAAAGGAGTGATCATGTCAG
RAA3	aattctaatacgactcactatagggTTGCGAAATCCTCAAGCTCAGTCAAAT ACACAAAGACCCCCAAAAAAGGAAAGAC
RT-PCR	TCTCTTgCgAAATCCTCAAgCT gAAAgACgCAAAggAgTgATCA

2.2. Methods

2.2.1. Screening of conserved gene sequences, plasmid synthesis, and extraction

Complete gene sequences of SFTSV strains were downloaded from Genbank. The library includes 573 sequences of the 6368-bp L segment, 515 sequences of the 3378-bp M segment, and 318 sequences of the 1744-bp S segment. It is generally believed that the sensitivity of nucleic acid amplification is highest in the S-segment, so Mega-X and NCBI-BLAST were used to conduct bioinformatics analysis of conserved sequences among the 318 full-gene S-segments. Based on the analysis results, the conserved sequence with GenBank ID: HQ141612.1 (1620–1744 bp) was selected for amplification. The conserved S segments of Hantavirus, influenza A virus (H1N1), Japanese encephalitis virus (JEV), dengue fever virus (DENV), tick-borne forest encephalitis virus (TBEV), and other specific pathogen controls(Table 1) were all selected in the same way. Conserved sequences were then integrated into plasmids with ampicillin resistance, and mixed with individual bacterial cultures (in 50 μL glycerol). Bacteria were amplified in 5 mL LB at 37 °C overnight. Plasmids were isolated using a plasmid extraction kit according to the manufacturer's instructions .

Templates for *in vitro* crRNA transcription were synthesized based on the known SFTSV crRNA sequences (Table 1). First, the primers and templates in Table 1 were used for PCR amplification. The reaction mixture included100 μL of 2 × Super pfx Master Mix, 8 μL (200 nM) of each upstream primer, downstream primer, and template, and 76 μL ddH_2O (for a total volume of 200 μL). The PCR reaction conditions were 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and a final extension step at 72 °C for 10 min. Reaction products were stored at 4 °C until use. The crRNAs were then transcribed *in vitro* using a T7 transcription kit, purified by magnetic beads, and stored at −80 °C for later use.

2.2.3. RAA synthesis

The SFTSV RAA was generated using an RT-RAA nucleic acid amplification kit according to the manufacturer's instructions. Briefly, the 50 μL reaction volume contained 41.5 μL of kit A Buffer, 2 μL (200 nM) of each upstream primer, downstream primer, and template (Table 1), and 2.5 μL of kit B Buffer. The mixture was shaken and incubated in a metal bath at 39 °C for 30 min. The product was extracted by phenol/chloroform, verified by electrophoresis on 2% agarose gels, and stored at 4 °C for later use.

2.2.4. CRISPR-based fluorescence detection of viral RNAs

The fluorescent CRISPR reaction system included 2 μL NTP MIX, 1 μL Inhibitor, 1 μL (240 mg/L) Cas13a, 0.5 μL T7, 0.25 μL (1 M) $MgCl_2$, 0.5 μL (1 M) HEPES, 10.75 μL ddH_2O, 2.5 μL (200 nM) reporter RNA, 1.5 μL (100 ng/μL) crRNA, and 5 μL template. The reaction system was mixed and fluorescence measured every 2 min for 30 min using a quantitative PCR instrument.

2.2.5. CRISPR-based detection of viral RNAs using test strips

The CRISPR reaction system for test strip detection contained 4 μL NTP MIX, 2 μL Inhibitor, 2 μL (240 mg/L) Cas13a, 1 μL T7, 0.5 μL (1 M) $MgCl_2$, 1 μL (1 M) HEPES, 26.5 μL ddH_20, 5 μL (400 nM) Cas probe, 3 μL (100 ng/mL) crRNA, and 5 μL template. The reaction system was mixed and reacted in a constant temperature metal bath at 37 °C for 30 min. A test strip was then inserted into the reaction tube for 1–2 min and the result (positive or negative) recorded by photography. The test strip quality control line (C line) and the test line (T line) can exhibit red bands. If the test strip T line has red bands and no matter C line show color or not, a positive result is indicated because Cas13a was able to effectively cut and activate the reporter group. Alternatively, if the quality C line shows red bands and the T line does not, the result is negative as Cas13a failed to cut the reporter molecule and could not activate the reporter group.

2.2.6. Evaluation of sensitivity and specificity

Sensitivity was measured using 10-fold serial dilutions starting with the SFTSV national reference product according to supplier instructions. Tests were conducted as described (Sections 2.3 and 2.4). For assessment of specificity, 5 negative reference materials (N1–N5) were subjected to nucleic acid extraction and CRISPR detection, and the results recorded. The five viral plasmids in Table 1 were also amplified and tested as described.

2.2.7. RT-PCR detection

PCR amplification was conducted using 2 × Taq PCR MasterMix reagent according to the manufacturer's instructions (Tiangen Biochemical Technology Company). The reaction mixture contained 1 μL (200 nM) template, 1 μL (200 nM) each of upstream and downstream primer, 12.5 μL of 2 × TaqPCR Mix, and 25 μL ddH_2O. Reaction conditions were as follows: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 10 min. Products were confirmed by agarose gel electrophoresis.

2.2.8. Sample nucleic acid extraction

The RNA of SFTSV national reference products was extracted by nucleic acid extraction kit (Qiagen, 52,904). In brief, 28 μL sample was mixed with 112 μL buffer AVL-carrier RNA and placed at room temperature for 10 min. After mixed with 560 μL anhydrous ethanol, the solutionto was added to column which was put into the centrifugal tube, and then centrifuged at 8000 rpm for 1 min. Added 500ul buffer AW1 and centrifuged at 8000 rpm for 1 min. Added 500ul buffer AW2 and centrifuged at 14,000 rpm for 3 min. Added 60ul buffer AVE and placed for 1 min, centrifuged at 8000 rpm for 1 min. Collected the final centrifugal fluid and stored in −20 °C. The DNA of synthetic plasmids was extracted by DNA extraction kit (Sangon Biotech, B518191-0050) in strict accordance with the kit instructions.

2.2.9. Agarose gel electrophoresis detection

Preliminary RAA screening and RT-PCR results were confirmed by gel electrophoresis using 2% agarose gels. Gels were produced by mixing 2.0 g agarose with 100 mL TAE electrophoresis solution, heating to a boil, cooling to ~60 °C, adding 5 μL nucleic acid dye, mixing thoroughly, evenly pouring the mixture into a gel plate, and allowing 30 min for cooling and setting. Mixed sample with loading buffer and added to sample tank. Electrophoresis was performed at 110 V and more than 40 mA. The results were photographed and observed under UV.

2.2.10. Treatment of standards and plasmids

The mass concentration of plasmid was measured by Nanodrop Uv-Vis spectrophotometers and the number of copies per microliter was calculated by molecular weight of plasmid. All plasmids were eventually diluted to 10^6 copies/μl as the corresponding template. The synthetic plasmids with the sequence of SFTSV (Table 1) was used as the template of the tests in Fig. 2A–D and the synthetic plasmids with the sequence of SFTSV, H1N1, HV, TBEV, JEV, DENV (Table 1) were used as the template of the tests in Fig. 4D–F. According to the official instructions, the initial concentrations of the positive national standards are, respectively 6.37×10^3 copies/μl (P1), 3.47×10^3 copies/μl (P2), 1.33×10^3 copies/μl (P3), 2.72×10^3 copies/μl (P4), 2.78×10^3 copies/μl (P5), 4.83×10^3 copies/μl (P6), and they were diluted 100 times with virus diluent (MEM medium +2% bovine serum) as the targets of tests in Figs. 2E–G, 4A–C and 5A. The initial concentrations of the negative national standards are, respectively 5.56 lg PFU/ml (N1), 5.56 lg PFU/ml (N2), 7.3 lg $CCID_{50}$/ml (N3), 6.09 lg PFU/ml (N4), 8.0 lg LD_{50}/ml (N5), and they were used as the target of test in Fig. 4A–C. Another tube of P6 with initial concentration was diluted to 1×10^3 copies/μl with virus diluent and then was serially diluted 10-fold to become sensitivity test sample with the concentration of 10^3, 10^2, 10^1, 10^0, 10^{-1} copy/μL in Figs. 3A–C and 5B. NC was no-template (i.e. no target) control.

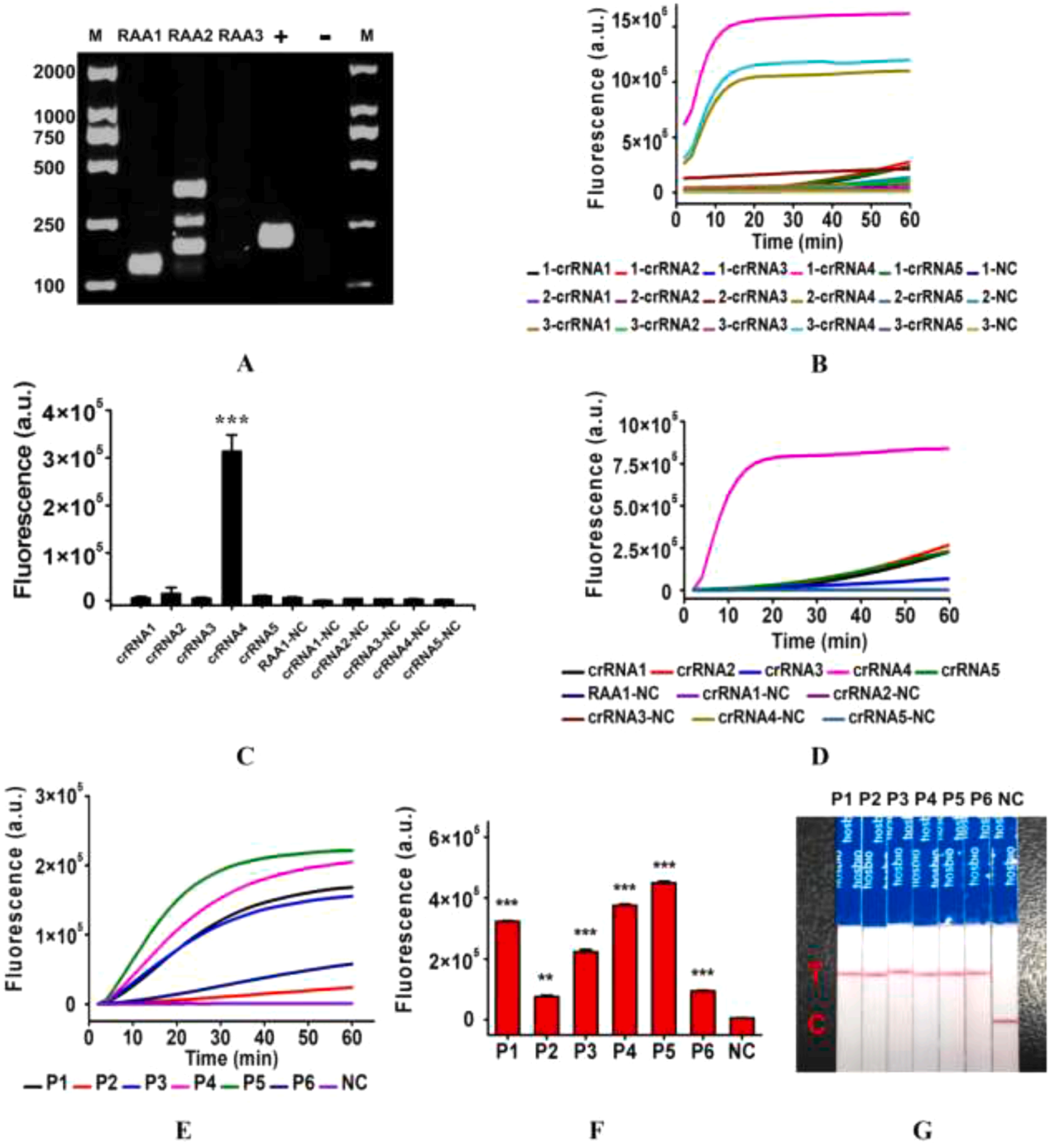


Fig. 2. Screening and evaluation of crRNA and RAA primers for target viral RNA detection.
(A) Agarose gel electrophoresis results for the three RAAs (RAA1–3). RAA1 demonstrates the most efficient and specific amplification. (B) Real-time CRISPR fluorescence detection results using different RAAs. (C) CRISPR fluorescence screening of crRNAs. crRNA-4 yielded the greatest emission. Intensity after 30 min (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to negative control [NC]). (D) Real-time fluorescence detection results using different crRNAs. (E) SFTSV positive reference product fluorescence results. (F) Fluorescence intensity of positive reference products after 30 min (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to NC). (G) SFTSV positive reference product test strip results. A colored lower line (C line) alone indicates a negative test while a colored upper line (T line) no matter with a colored C line or not both indicates the presence of SFTSV.

2.2.11. Statistical analysis

To ensure test accuracy and reproducibility, three independent technical replicates were conducted for CRISPR-based fluorescence detection, CRISPR-based test strips detection, agarose gel electrophoresis detection, RAA synthesis and RT-PCR detection. Datasets with equal variance were examined by one-way analysis of variance (ANOVA) and those with uneven variance by Dunnett and Tamhane nonparametric tests.

3. Results

3.1. Selection of RAA amplification primers and crRNA nucleic acid detection targets

We first compared the efficiency of RAA amplification to identify the most appropriate primer for the SFTSV test. In all cases, amplification was performed according to the template with a concentration of 10^6

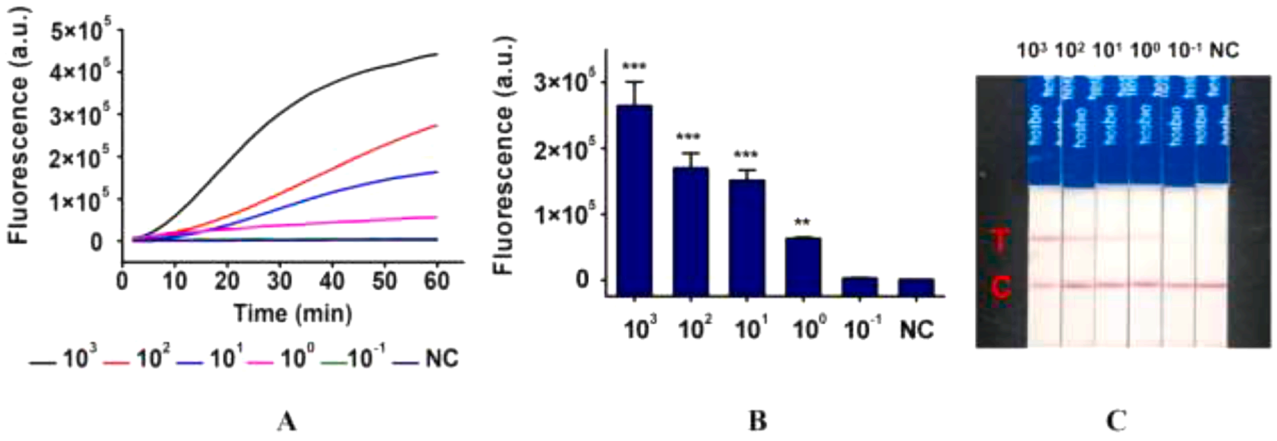


Fig. 3. Sensitivity of CRISPR-based SFTSV detection
(A) CRISPR-based fluorescence emission from serially diluted SFTSV RNAs. (B) Fluorescence intensity after 30 min (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to NC). (C) CRISPR paper test detection of SFTSV RNA at different starting concentrations. A colored lower line (C line) alone indicates a negative test while a colored upper line (T line) no matter with a colored C line or not both indicates the presence of SFTSV.

copies/μl and results evaluated by gel electrophoresis (Fig. 2A). The negative control (NC) lane exhibited no bands, indicating that the amplification process was pollution-free. Of the three primers, RAA3 generated no bands, RAA1 a single intense band indicative of efficient and specific amplification, and RAA2 multiple bands indicative of non-specific amplification. To confirm the greater efficiency and specificity of RAA1 over RAA2, CRISPR fluorescence detection was performed for each RAA primer and the 5 crRNAs, respectively (Fig. 2B). Consistent with electrophoresis results (Fig. 2A), the amplification efficiency of RAA1 was superior to RAA2. Further, both RAA1 and RAA2 demonstrated highest binding efficiency with crRNA4. To verify that crRNA-4 is the most effective target, fluorescence emission was compared among reactions using RAA1 and each of the 5 crRNAs. RAA1-NC was a no crRNA control, and crRNA1-NC to crRNA5-NC were no-template controls for each crRNA and primer tested (Fig. 2C and D). Again, crRNA-4 demonstrated the best amplification efficiency. At the 30th minute, the average fluorescence intensity of the crRNA-4 reaction was significantly higher than the other 4 reactions (799,236 a.u. vs. 46,271 a.u. for crRNA1, 61,196 a.u. for crRNA2, 27,767 a.u. for crRNA3, 65,443 a.u. for crRNA5, and 0 a.u. for the negative control), so crRNA-4 was selected as the nucleic acid detection target for subsequent experiments.

RAA1 and crRNA-4 were then used to detect the 6 positive national standards (P1–P6, SFTSV strain AH12, HN1, JS3, LN3, SD4, HB29), with fluorescence detection results presented in Fig. 2E, and F, and test strip results in Fig. 2G. At the 30th minute of fluorescence detection, emission intensity values were 240,799 a.u. (P1), 20,205 a.u. (P2), 228,474 a.u. (P3), 305,869 a.u. (P4), 385,306 a.u. (P5), and 51,777 a.u. (P6), all significantly greater than the negative control (800 a.u.), thus confirming the target specificities of RAA1 and crRNA-4 for CRISPR detection of SFTSV.

3.2. Sensitivity of CRISPR-based SFTSV nucleic acid detection

The nucleic acid extracted from the reference sample demonstrating highest sensitivity was serially diluted and subjected to CRISPR detection for systematic sensitivity testing (Fig. 3A and B). The fluorescence signals increased progressively with time and starting concentration ($10^3 > 10^2 > 10^1 > 10^0 > 10^{-1}$ copies/μL), with no fluorescence was detected at 10^{-1} copies/μL even after 30 min. Thus, we defined 10^0 as the threshold for detection. Similar results were found using test paper (Fig. 3C), with negativity yielded by both 10^{-1} copies/μL and the NC sample (the lower "C" line alone in Fig. 3C) while 10° copies/μL and above yielded a positive test (the "T" line in Fig. 3C).

3.3. Specificity of CRISPR-based SFTSV nucleic acid detection

To examine the specificity of CRISPR-based SFTSV detection, we compared the fluorescence signals and test strip readings from 5 negative SFTSV national reference products (N1–N5, Japanese encephalitis virus vaccine strain SA14-14-2, dengue virus type 4 strain Ban18, yellow fever virus strain 17D, HFRS VIRUS strain L99 and forest encephalitis virus strain Senzhang), each a known viral pathogen with similar transmission routes or causing similar symptoms to SFTSV, and NC samples (Fig. 4). Fluorescence intensity values of the negative samples N1–N5 after 30 min were significantly lower than that from P1 and not markedly different from the NC (Fig. 4A, B). All 5 negative reference products also gave negative readings on the test paper (all yielding a colored "C line" only) (Fig. 4C). Moreover, samples of Hantavirus (HV), H1N1, Japanese encephalitis (JEV), dengue virus (DENV), and tick-borne forest encephalitis (TBEV) also yielded emission signals similar in magnitude to the NC and significantly lower than that from SFTSV (Fig. 4D–F). Also, all 5 pathogenic viruses yielded negative readings on paper strip tests, while an SFTSV sample gave a positive reading (colored "T" line). Collectively, these results demonstrate the specificity of both CRISPR-based tests for SFTSV.

3.4. Confirmation of CRISPR-based test results by RT-PCR

For further confirmation of test accuracy, results were compared to RT-PCR detection (Fig. 5A and B) of the 6 positive standards, sensitivity standards, and negative standards. Clear nucleic acid bands were detected on agarose gel lanes loaded with the 6 positive reference products, and detection was achieved at 1 copy per microliter. Thus, the sensitivity of the CRISPR-based test matched that of RT-PCR.

4. Discussion

Severe fever with thrombocytopenia syndrome is an acute infectious disease first described in central China during 2009. In the years since, several outbreaks of SFTS have occurred and the endemic region has expanded to include more than 20 provinces in China [(3)]. Cases have also been detected in South Korea, Japan, the United States, and other countries (Kim et al., 2015). The causative virus SFTSV mainly infects humans through tick bites and subsequent airborne transmission. Serious infections can damage multiple organs, and the mortality rate of hospitalized patients can be as high as 10%–30% (Jianbo et al, 2017; Wang et al., 2014).

At present, there are many detection methods for SFTSV, but all are

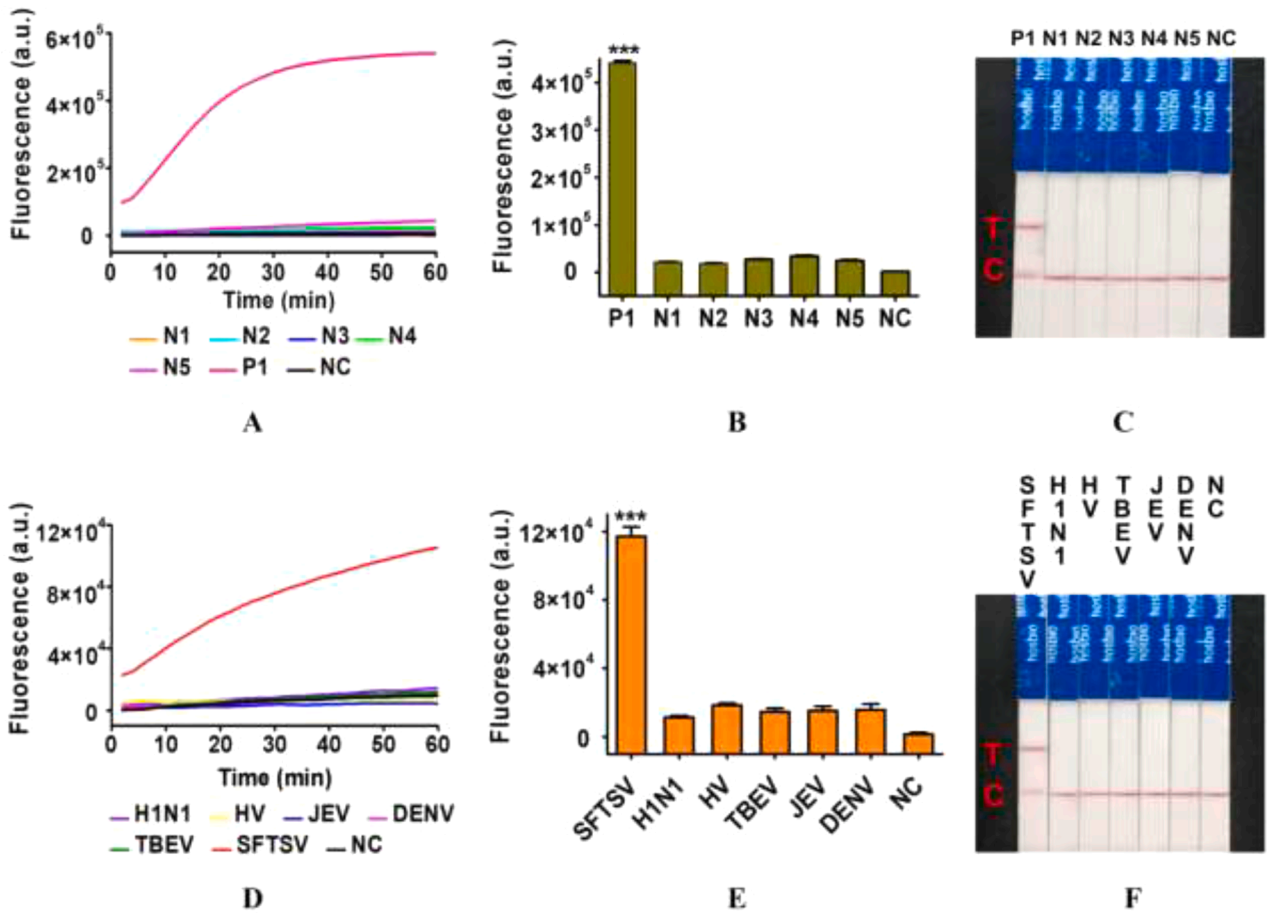


Fig. 4. Specificity of CRISPR-based SFTSV detection
(A) CRISPR-based fluorescence emissions from negative reference products (N1–N5) compared to a reference SFTSV sample (P1). (B) Fluorescence emission at 30 min for each sample (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to NC). (C) Corresponding paper strip test results. (D) CRISPR-based fluorescence emissions from non-SFTSV pathogenic viruses and a SFTSV sample. E. Fluorescence at 30 min (*$P < 0.05$,**$P < 0.01$,***$P < 0.001$ compared to NC). (F) Corresponding paper strip test results. A colored lower line (C line) alone indicates a negative test while a colored upper line (T line) no matter with a colored C line or not both indicates the presence of SFTSV.

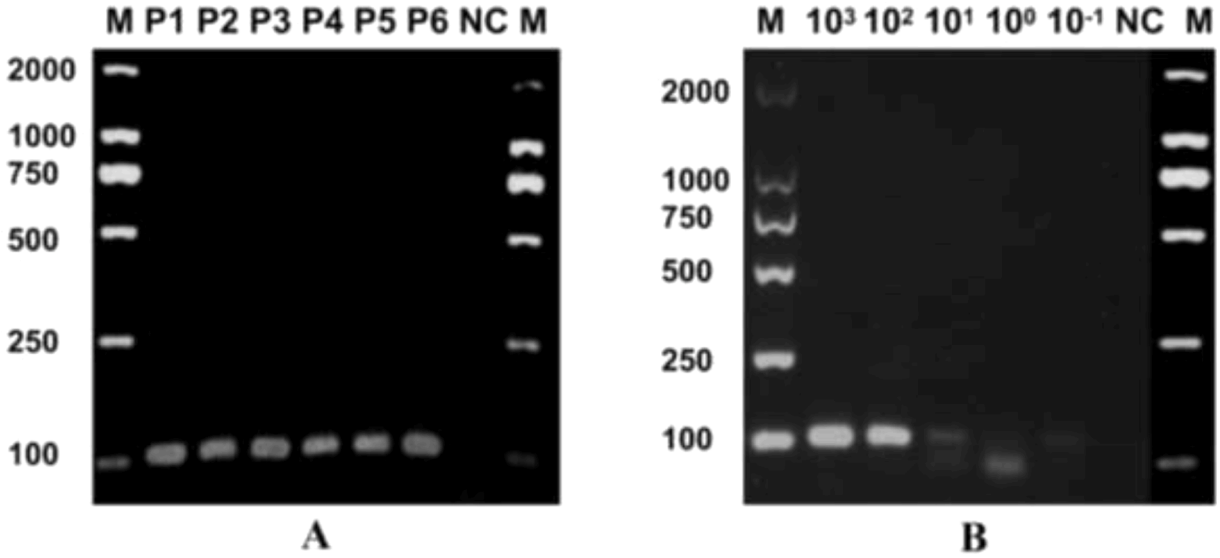


Fig. 5. RT-PCR detection of SFTSV results
(A) Detection of SFTSV positive reference product by RT-PCR. (B) SFTSV sensitivity of RT-PCR.

either insufficient, time-consuming, costly, or of limited accuracy. Du and colleagues first described a virus isolation method for SFTSV. While sensitive, it requires about 2 weeks to culture the serum samples (or even longer if the virus content is low) (Hu and Bao, 2018). Nucleic acids in biological samples are detected mainly by PCR. For instance, Li and colleagues achieved a $TCID_{50}$ of 10^{-4} and results within two hours, but the supporting equipment is expensive and possible unavailable in rural areas (Li et al., 2012). An ELISA test for SFTSV has also been developed, but it too requires expensive fluorescence microplate readers or microscopes among other instruments, and there are strict requirements for the testing environment (Pang and Li, 2013a). Infections occur mainly in areas with poor medical and health infrastructure, so samples must be collected by primary health institutions and shipped to central institutions with testing capabilities. During transportation, the virus nucleic acid may be degraded, which severely reduces diagnostic accuracy. Therefore, a portable, fast, and accurate on-site test is urgently needed.

A variety of rapid pathogen detection methods have been developed in recent years for disease diagnosis. Point of care tests (POCTs) must be portable and rapid as well as accurate. Hu and colleagues (Hu et al., 2014) developed a surface plasmon resonance (SPR) biological analysis system able to detect porcine circovirus type 2 (PCV-2) in 20 min with a detection limit of 0.04 mg/mL. Shelby et al. (2016) and colleagues used electromagnetic methods to detect and distinguish various influenza viruses, and detected influenza glycoproteins at concentrations as low as 1 nmol/L within 30 min. Grabowska et al. (2014) used electrochemistry to detect influenza A H5N1 virus at concentrations as low as 0.08 fmol/L. For prevention and control of COVID-19, Joung et al. (2020) introduced a CRISPR-based STOP (Sherlock Testing In One Pot) method that can identify positive samples in about 30 min. Similar CRISPR-based systems have shown a high degree of specificity and performance for detecting SARS-CoV-2 (Joung et al., 2020), Zika virus (Pardee et al., 2016), HPV (Zhang et al., 2018a; Wang et al., 2018; Zhang et al., 2018b), and Mycobacterium tuberculosis (Zhang et al., 2017). The feasibility of combining CRISPR-based methods with other technologies should facilitate the development of many such pathogen detection tests for future epidemics and pandemics.

In this study, we combined CRISPR-cas13a isothermal amplification RAA with colloidal immunochromatography to produce a rapid on-site strip test requiring only a biological sample and a simple temperature control system (as both CRISPR and RAA reactions can be conducted at the same constant temperature of 37 °C). Further, the entire reaction time is only 150 min, significantly shorter than for RT-PCR, and is also much quicker than virus isolation and ELISA. This first iteration demonstrated a minimum detection limit of 10° copies/μL, a level of sensitivity similar to RT-PCR. When combined with test paper, results could be visualized in 1–2 min. The specificity of this assessment was confirmed using 5 negative reference viruses provided with the SFTSV national reference materials as well as by testing for multiple viruses of the same genus (Hantavirus) causing similar syndromes (H1N1 influenza virus), or that are also tick-borne (JEV, DENV, TBEV). Therefore, this test will permit differential diagnosis of patients with severe fever in regions where tick-borne infections are endemic.

Continued advances in CRISPR detection technology have led to the development of rapid and convenient tests applicable in regions without advanced high-throughput detection equipment. Other CRISPR-based nucleic acid detection technologies include CRISPR/Cas9-mediated lateral flow nucleic acid analysis (CASLFA) first established by Wang et al., which can detect as few as several hundred copies of African swine fever virus (ASFV) in one hour (Wang et al., 2020), and the CRISPR-mediated DNA-FISH method incorporating a SYBR Green I(SG I) fluorescent probe (Zhang et al., 2018b), which can detect as little as 10 cfu/mL methicillin-resistant Staphylococcus aureus (MRSA) in 30 min (Guk et al., 2017). Other CRISPR based methods can further accelerate diagnosis by detection of the target virus directly from biological samples. For instance, a pathogen sample processing method termed Hudson (heating the unextracted diagnostic sample to eliminate nuclease) can detect ZIKV and Dengue virus in patient samples (such as serum, urine, and saliva) within two hours at concentrations as low as 1 copy per microliter without nucleic acid extraction (Myhrvold et al., 2018). An enhanced SHERLOCKv2 has also been described in which four different Cas13 and Cas12 enzymes are combined for detection, and four nucleic acid sequences can be detected in one reaction. The activity of Cas13 was also increased by using CRISPR-related enzyme Csm6 (Kazlauskiene et al., 2017), yielding a 3.5-fold increase in detection sensitivity (Gootenberg et al., 2018). In addition to RAA, CRISPR can be combined with many other nucleic acid amplification methods for pathogen detection. Detection combining CRISPR and loop-mediated isothermal amplification (LAMP) has been described, but requires the design of multiple pairs of primers and targets to improve accuracy (Grabowska et al., 2014). The combination of CRISPR and PCR (Zhang et al., 2018b) can improve accuracy, but increases the complexity of detection. In our study, the visual detection of SFTSV based on CRISPR and RAA is fast, sensitive, and portable for rapid on-site detection.

One limitation of this study is that a commercial nucleic acid extraction kit was used without optimization. Second, the tests uses separate amplification and CRISPR reaction steps, which may be unnecessary, as a recently reported method combined LAMP and CRISPR reactions into one step for COVID-19 detection (Joung et al., 2020). Therefore, it is possible to optimize the reaction system so that nucleic acid amplification and detection are conducted simultaneously at the same temperature, further improving the efficiency of on-site detection. Further, multiple targets or multiple Cas enzymes could be included to enhance detection sensitivity.

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