Original article

Rapid and visual detection of 2019 novel coronavirus (SARS-CoV-2) by a reverse transcription loop-mediated isothermal amplification assay

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ABSTRACT

Objective: To evaluate a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and compare it with RT-PCR.

Methods: We designed primers specific to the *orf1ab* and *S* genes of SARS-CoV-2. Total viral RNA was extracted using the QIAamp Viral RNA Mini Kit. We optimized the RT-LAMP assay, and evaluated it for its sensitivity and specificity of detection using real-time turbidity monitoring and visual observation.

Results: The primer sets orf1ab-4 and S-123 amplified the genes in the shortest times, the mean ($\pm$SD) times were 18 ± 1.32 min and 20 ± 1.80 min, respectively, and 63°C was the optimum reaction temperature. The sensitivities were 2×10^1 copies and 2×10^2 copies per reaction with primer sets orf1ab-4 and S-123, respectively. This assay showed no cross-reactivity with 60 other respiratory pathogens. To describe the availability of this method in clinical diagnosis, we collected 130 specimens from patients with clinically suspected SARS-CoV-2 infection. Among them, 58 were confirmed to be positive and 72 were negative by RT-LAMP. The sensitivity was 100% (95% CI 92.3%−100%), specificity 100% (95% CI 93.7% −100%). This assay detected SARS-CoV-2 in a mean ($\pm$SD) time of 26.28 ± 4.48 min and the results can be identified with visual observation.

Conclusion: These results demonstrate that we developed a rapid, simple, specific and sensitive RT-LAMP assay for SARS-CoV-2 detection among clinical samples. It will be a powerful tool for SARS-CoV-2 identification, and for monitoring suspected patients, close contacts and high-risk groups. **C. Yan, Clin Microbiol Infect 2020;26:773**

Introduction

The outbreak of a cluster of respiratory infections designated as coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), had a significant impact on both the health and economy of China [1—4]. To date (1 April 2020), up to 754 948 SARS-CoV-2 cases have been confirmed, including more than 36 571 deaths (https://www.who.int/home). Human-to-human transmission was confirmed through a case of five patients in a family cluster [5]. The virus transmitted rapidly via aerial droplets, contact and fomites [6].

SARS-CoV-2 has been classified as a β-coronavirus of group 2B with higher similarity, over 96% identical at the whole-genome level, to two bat-derived coronavirus strains than to other known human coronaviruses [7,8]. The genome of the novel virus comprises a 5′ untranslated region, replicase complex (*orf1ab*), Spike

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gene (*S* gene), *E* gene, *M* gene, *N* gene, 3′ untranslated region, and several unidentified non-structural open reading frames [9].

Since the outbreak of SARS-CoV-2 infection, real-time RT-PCR assays have played an important role in clinical diagnosis and the investigation of suspected cases. However, such methods are laborious, time-consuming and require specialized instruments, and are therefore not able to satisfy the current rapid growth and demands of testing the large number of suspected patients, asymptomatic patients and close contacts. A rapid, simple, and sensitive assay is urgently needed [10].

Loop-mediated isothermal amplification (LAMP) was developed by Notomi et al. in 2000 [11]. It is a rapid, sensitive and effective visual nucleic acid amplification method. Recently, this method has been widely applied for the detection of influenza virus, Middle East respiratory syndrome-CoV, West Nile virus, Ebola virus, Zika virus, yellow fever virus and a variety of other pathogens [12–17]. The test relies on auto-cycling strand displacement DNA synthesis using four to six specific primers to recognize six to eight sequences of the target gene in only 1 hour.

In this study, we developed a reverse transcription LAMP (RT-LAMP) assay to detect SARS-CoV-2 in individuals with COVID-19. We designed five sets of primers that target the *orf1ab* gene and the *spike* gene for optimization of the assay. We also evaluated the sensitivity and specificity of this method. Finally, 130 clinical samples collected from patients were analysed for clinical diagnosis.

Materials and methods

Viruses and bacterial strains

A total of 60 respiratory pathogens including human coronavirus HCoV-229E, HCoV-NL63, HCoV-OC43, HCoV-HKU1, SARS-CoV (pseudo-virus) and Middle East respiratory syndrome-CoV (pseudo-virus); influenza A-H3N2, H1N1, influenza B, parainfluenza viruses types 1/2/3/4, adenoviruses types 1/2/3/4/5/6/7, respiratory syncytial virus types A/B, human metapneumovirus, human bocavirus, rhinovirus types A/B/C, as well as respiratory pathogens such as *Mycoplasma pneumoniae* strains M129/FH, *Haemophilus influenzae* ATCC 49247, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* were used in this study.

RNA extraction

Total viral RNA was extracted from the specimens using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The extracted RNAs were stored at −80°C.

Preparation of the artificial SARS-CoV-2 gene

The full-length 3822-bp *spike* gene (21 563–25 384) and a 500-bp sequence (13 791–14 290) of the *orf1ab* gene of SARS-CoV-2 (GenBank Accession No. MN908947.3) were synthesized (Sangon Biotec Co., Ltd., Shanghai, China) and cloned into vector pUC57. Pseudo-viruses of 2019-nCov-S and 2019-nCov-ORF1ab (Xiamen Zeesan Biotech Co., Ltd, Xiamen, China) were also used. The copy number of the pseudo-viruses was calculated using the following formula: copies/µL = $6.02 \times 10^{23} \times 10^{-9} \times$ concentration (ng/µL)/ (fragment length × 340). Then, ten-fold serial dilutions of the pseudo-viruses ranging from 1×10^8 copies/µL to 1 copy/µL were prepared.

Primer design

We aligned 103 complete genomes of SARS-CoV-2 obtained from four databases, GenBank, GISAID, GWH and NMDC (https://bigd.big.ac.cn/ncov), to confirm the conservation of the selected sequence. Homology analysis was conducted using BLAST (National Centre for Biotechnology Information). Five sets of primers specific to the *orf1ab* and *S* genes were designed using the software PRIMEREXPLORER V5 at the website: http://primerexplorer.jp/e/. The primers included an outer forward primer (F3), an outer backward primer (B3), a forward inner primer (FIP) and a backward inner primer (BIP). A loop forward primer (LF) and/or a loop backward primer (LB) were also designed to accelerate the reaction. We also designed primers to amplify the two regions by conventional PCR assay (orf1ab-F: 5′-CAGACCTCGTCTATGCTTTAAGGC-3′; orf1ab-R: 5′-CCCTGGTCAAGGTTAATATAGGCA-3′; S–F: 5′-CTTCCCTCAGTCAG-CACCTC-3′; S-R: 5′-AACCAGTGTGTGCCATTTGA-3′). The primers were synthesized by Shanghai Invitrogen Biological Engineering, Co., Ltd (Shanghai, China) and purified by high-performance liquid chromatography.

RT-LAMP assay

A Loopamp RNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan) was used to perform the RT-LAMP reaction. The reaction volume of 25 µL contained 2 × Reaction Mix 12.5 µL, Enzyme Mix (a mix of *Bst* DNA polymerase and AMV reverse transcriptase) 1.0 µL, primers FIP and BIP (40 pmol), primers F3 and B3 (5 pmol), primers LF and/or LB (20 pmol), fluorescent detection reagent 1 µL (when needed), and template RNA 2 µL. The mixture was incubated at 63°C for 60 min. The process was monitored using a Loopamp Real-time Turbidimeter (LA-230; Eiken Chemical Co., Ltd., Tochigi, Japan). Turbidity readings at an optical density at 650 nm were obtained every 6 seconds, and the reaction was considered positive when the turbidity values were >0.1. For visual observation, 1 µL of fluorescent calcein was added to the mixture, and a colour change from orange to green was observed by the naked eye for a positive reaction.

Real-time RT-PCR assay

A commercial real-time RT-PCR kit (BGI PathoGenesis Pharmaceutical Technology, Shenzhen, China) was used as the reference standard for the RT-LAMP. The total reaction volume was 30 µL and the reaction was set up according to the manufacturer's protocol. The reaction procedure was 50°C for 20 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. A threshold cycle (*Ct* value) < 38 was determined to indicate a positive sample.

Clinical specimens

One hundred and thirty clinical specimens were collected from individuals with pneumonia and suspected SARS-CoV-2 infection, including swabs and bronchoalveolar lavage fluid. Initial processing of all specimens was handled in a validated biological safety cabinet, and performed by trained staff wearing appropriate personal protective equipment. The extracted RNA was divided into two parts for amplification. The RT-LAMP assays and real-time RT-PCR were performed simultaneously in a biosafety level 2 laboratory, as detailed in the WHO Laboratory biosafety manual, third edition. The amplified products of the RT-LAMP were detected by turbidity monitoring as well as visual observation. To assess turbidity, the time of the positivity detection was counted. Mean and standard deviation were used to describe the data and 95% CI were presented where relevant. This work was approved by the research board of

the Ethics Committee of the Capital Institute of Paediatrics, Beijing, China. All patients provided informed consent in accordance with the Declaration of Helsinki.

Results

Optimizing the RT-LAMP assay

The conserved regions of the *orf1ab* gene and *S* gene of SARS-CoV-2 with low mutation frequencies were candidate target sequences (see Supplementary material, Fig. S1). The pseudo-viruses were used as the template (concentration 1 ng/μL) to optimize the RT-LAMP reaction conditions. Five sets of primers were designed to respectively detect the *orf1ab* gene (orf1ab-212, orf1ab-4, orf1ab-33, orf1ab-22 and orf1ab-1) and the *spike* gene (S-107, S-123, S-67, S-41 and S-61) of SARS-CoV-2 using a real-time LAMP turbidimeter. As shown in Fig. 1, the primer sets orf1ab-4 and S-123 amplified the genes in the shortest times, the mean (±SD) times were 18 ± 1.32 min and 20 ± 1.80 min, respectively, so these two primer sets were chosen as the optimal primers for RT-LAMP detection. All of the primer sets used in this study are listed in the Supplementary material (Table S1).

To screen for the optimum temperature, the reaction was incubated at six different temperatures (60°C–65°C) for 60 min. As

presented in Fig. 1, the highest amplification efficiency occurred at 63°C. Therefore, 63°C was confirmed as the optimum reaction temperature for this assay.

Sensitivity test for the RT-LAMP assay

The sensitivity of the RT-LAMP assay using primer sets orf1ab-4 and S-123 was evaluated using turbidity monitoring and visual observation. Ten-fold serial dilutions of the pseudo-viruses, ranging from 1×10^8 copies/μL to 1 copy/μL (concentration of template input) were detected by the RT-LAMP assays. As illustrated in Fig. 2, the time taken for positive detection ranged from 18 min at 1×10^8 copies/μL to 48 min at 1×10^1 copies/μL using primer set orf1ab-4, whereas for primer set S-123, the time taken for positive detection ranged from 21 min at 1×10^8 copies/μL to 50 min at 1×10^2 copies/μL. Hence, sensitivity of the assays was 2×10^1 copies and 2×10^2 copies per reaction at 63°C within 60 min with primer sets orf1ab-4 and S-123, respectively. The detection limit was the same with visual observation, with all positive reactions changing to green and negative reactions remaining orange as observed by the naked eye. But sensitivity of the conventional PCR was 2×10^2 copies and 2×10^3 copies per reaction with primer sets targeting orf1ab gene and S gene, respectively.
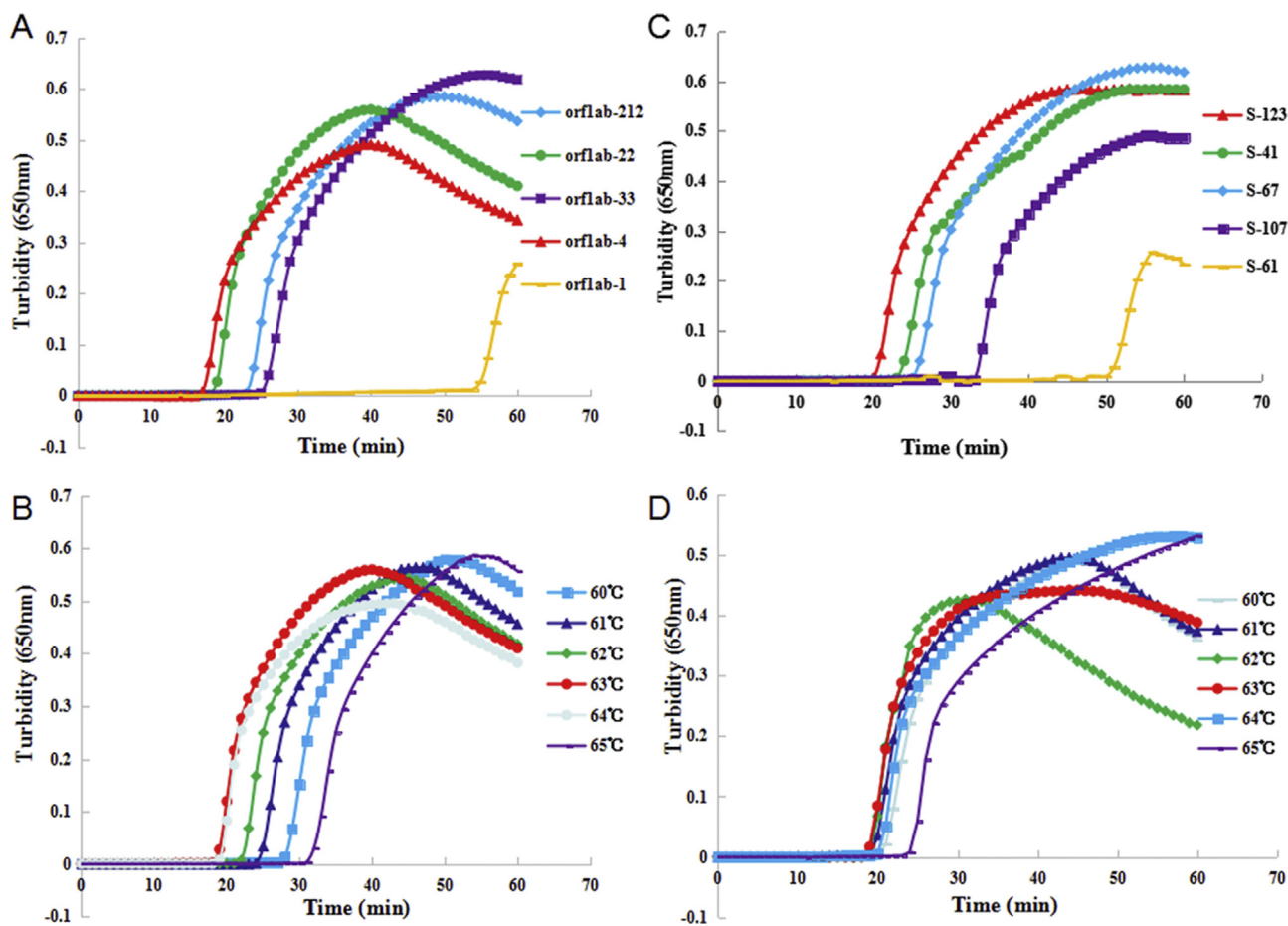

Fig. 1. The most appropriate primers and reaction temperature for the reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay. (A) Most suitable primer set for the RT-LAMP assay to amplify the *orf1ab* gene. (B) Optimal reaction temperature for the RT-LAMP assay with primer orf1ab-4. (C) Most suitable primer set for the RT-LAMP assay to amplify the *S* gene. (D) Optimal reaction temperature for the RT-LAMP assay with primer S-123. The reaction volume was 25 μL contained 2 μL RNA template, and the template concentration was 1 ng/μL.

Specificity test for RT-LAMP assay

The specificity of the RT-LAMP assays was evaluated using 60 strains of human respiratory pathogens. Fig. 3 reveals that only the pseudo-viruses tested positive, the nucleic acids of other respiratory pathogens and the blank control all tested negative by the RT-LAMP assay with primer sets orf1ab-4 and S-123, using turbidity monitoring and visual observation. Therefore, the RT-LAMP assay showed no cross-reactivity with other respiratory pathogens.

Clinical sample detection

The 130 swabs and bronchoalveolar lavage fluid samples obtained from individuals with pneumonia and suspected SARS-CoV-2 infection were simultaneously investigated by RT-LAMP with the two primer sets (orf1ab-4 and S-123) and by real-time RT-PCR. Among them, 58 individuals were confirmed to be infected with SARS-CoV-2, while 72 tested negative (Table 1). The sensitivity was 100% (95% CI 92.3%–100%) and specificity was 100% (95% CI 93.7%–100%)

Discussion

This study established a rapid, sensitive and specific assay for SARS-CoV-2 detection by RT-LAMP. To improve the sensitivity, we chose two target genes, orf1ab and S, to detect viral RNA in clinical samples. To ensure the stability of the detection assay, we aligned 103 complete virus genomes, then selected conversed regions without gene mutations in the orf1ab (245 bp) and S (216 bp) genes. To reduce the reaction time, we designed loop primers. Briefly, six primers targeting eight regions generated a self-priming dumbbell-shaped template upon isothermal incubation with strand-displacing polymerase, resulting in the rapid production of large quantities of complex amplicon [18].

Since the release of the first SARS-CoV-2 genome sequence via the community online resource, virological.org, on 10 January (Wuhan-Hu-1, GenBank Accession no.: MN908947), more than 2500 coronavirus sequences have been uploaded to date. These data make it possible for researchers to develop assays to detect viral RNA, to quarantine the source of infection, to explore the origin and native host(s), and to reveal the likely receptor-binding properties of this novel coronavirus.
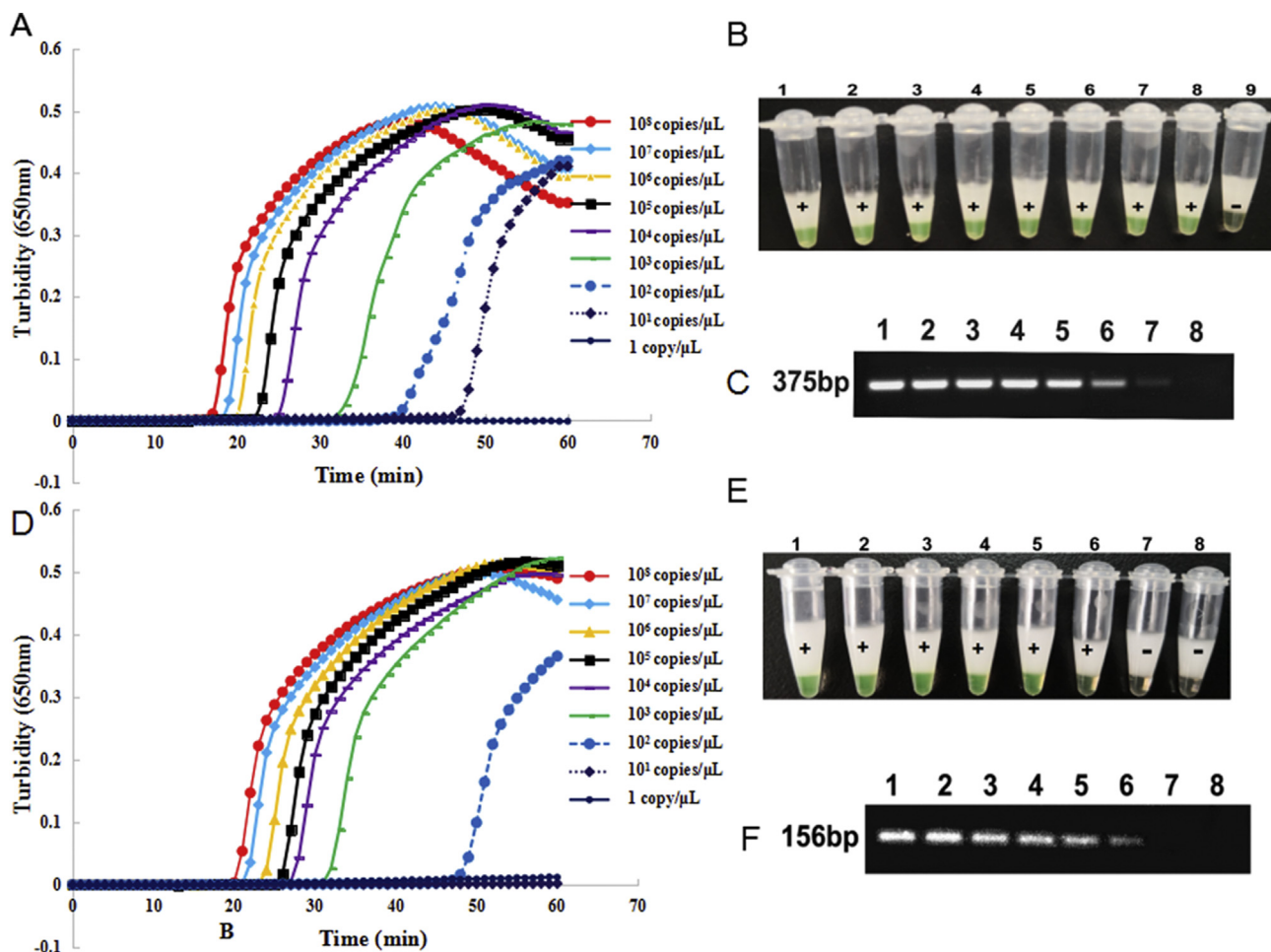

Fig. 2. Sensitivity of the reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay and conventional PCR for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection. (A,B) Sensitivity of the RT-LAMP assay using primer set orf1ab-4 to target the orf1ab gene for SARS-CoV-2 detection. (D,E) Sensitivity of the RT-LAMP assay using primer set S-123 to target the S gene for SARS-CoV-2 detection. (C,F) Sensitivity of the conventional PCR assay targeting the orf1ab and S genes for SARS-CoV-2 detection. The detection was monitored by turbidity using a Loopamp real-time turbidimeter, and was judged by the naked eye depending on a colour change from orange to green. (B), Lanes 1–9: 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 copies/μL; (E) lanes 1–8: 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 copies/μL; (C) and (F) lanes 1–8: 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 copies/μL. In the sensitivity test, 60 min can be used as the cut-off for the visual detection.
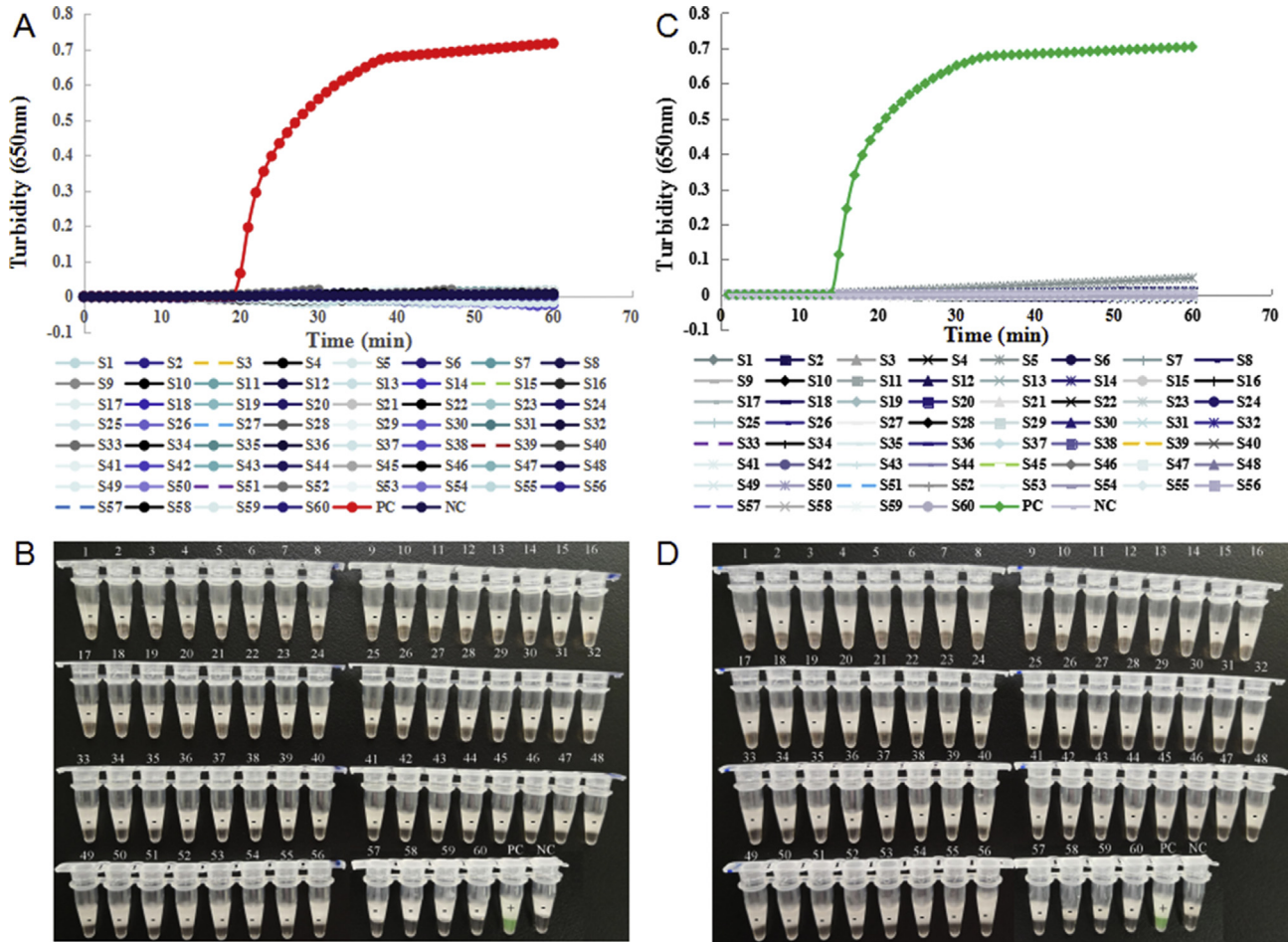
Fig. 3. Specificity of the reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection. (A,B) Specificity of the RT-LAMP assay with primer set orf1ab-4 targeted the *orf1ab* gene for SARS-CoV-2 detection. (C,D) Specificity of the RT-LAMP assay with primer set S-123 targeted the *S* gene for SARS-CoV-2 detection. The detection was monitored by turbidity using a Loopamp real-time turbidimeter (A, C), and was judged by the naked eye depending on a colour change from orange to green (B, D). S1: human coronavirus (HCoV) -229E-1; S2: HCoV-229E-2; S3: HCoV-NL63-1; S4: HCoV-NL63-2; S5: HCoV-NL63-3; S6: HCoV-OC43-1; S7: HCoV-OC43-2; S8: HCoV-HKU1-1; S9: HCoV-HKU1-2; S10: H3N2-1; S11: H3N2-2; S12: H3N2-3; S13: H3N2-4; S14: H1N1-1; S15: H1N1-2; S16: influenza B-1; S17: influenza B-2; S18: influenza B-3; S19: parainfluenza (PIV) -1-1; S20: PIV-1-2; S21: PIV-2-1; S22: PIV-2-2; S23: PIV-3-1; S24: PIV-3-2; S25: PIV-4-1; S26: PIV-4-2; S27: adenovirus (ADV) -1-1; S28: ADV-1-2; S29: ADV-2-1; S30: ADV-2-2; S31: ADV-3-1; S32: ADV-3-2; S33: ADV-4; S34: ADV-5-1; S35: ADV-5-2; S36: ADV-6; S37: ADV-7-1; S38: ADV-7-2; S39: respiratory syncytial virus (RSV) A-1; S40: RSV A-2; S41: RSV B-1; S42: RSV B-2; S43: human metapneumovirus HMPV-1; S44: HMPV-2; S45: human bocavirus (BoV) -1; S46: BoV-2; S47: rhinovirus (Rh) A-1; S48: Rh A-2; S49: Rh B-1; S50: Rh B-2; S51: Rh C; S52: *Mycoplasma pneumoniae* (MP) -FH; S53: MP-M129; S54: *Haemophilus influenzae*; S55: *Staphylococcus aureus*; S56: *Klebsiella pneumoniae*; S57: *Streptococcus pneumoniae*; S58: *Pseudomonas aeruginosa*; S59: SARS-CoV; S60: Middle East respiratory syndrome-CoV; PC, positive control (pseudo-virus); NC, negative control (distilled water).

The novel RT-LAMP assay presented positive results in the mean ($\pm$SD) time of 26.28 $\pm$ 4.48 min; whereas the RT-PCR assay requires 1–2 h after viral RNA preparation [19]. The sensitivity of this assay was 20 copies per reaction, similar to that reported for the RT-PCR assay in a previous study [20]. The RT-LAMP assay showed no cross-reactivity with other respiratory pathogens, so the diagnostic specificity of this method was higher than that reported for the serology test [21].

To assess the applicability of the assay for the clinical diagnosis of SARS-CoV-2 infection, an evaluation of 130 clinical samples was conducted. Sensitivity and specificity were both 100%. In fact, compared with the RT-PCR method, the RT-LAMP is easy to handle,

Table 1
Comparison between the reverse transcription loop-mediated isothermal amplification assay and RT-PCR for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection from clinical samples

Results	RT-LAMP		Real-time RT-PCR
	orf1ab gene	*S* gene	
Positive samples	58	58	58 (Ct < 38)
Mean $\pm$ SD (min)	25.36 $\pm$ 5.09	27.21 $\pm$ 5.74	
Range[a] (min)	16–35	18–40	
Negative samples	72 (no change in colour)	72 (no change in colour)	72 (no peak)

RT-LAMP, reverse transcription loop-mediated isothermal amplification; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation.
[a] Range: the shortest detection time − the longest detection time.

and does not require skilled personnel or specialized instruments. The visual RT-LAMP assay can complete detection within 60 min, which was faster than RT-PCR assay, which required more than 80 min. In addition, the positive results were directly visible and so easier to observe.

Strengths of this study include that the RT-LAMP assay is an important point-of-care testing detection method. The results are easy to read. Positive results could be judged by the naked eye according to a colour change from orange to green, while negative results remained orange. Moreover, the designed RT-LAMP primers targeted five/six independent regions. In addition, the new method can detect the virus in a single step at a constant temperature of 63°C, so the assay can be performed using a small instrument at the bedside or in the field.

The main limitation of the current study is that we aligned only 103 complete genomes of SARS-CoV-2 obtained from four databases when designing the primers. With the spread of this virus, the accuracy of this RT-LAMP assay will be affected by mutations occurring in the primer sequence region of the target gene. So, it is necessary to monitor the mutant sites of the virus genome by whole-genome sequencing.

The established RT-LAMP assay has important implications for clinical practice. COVID-19 is widely distributed throughout 203 countries, areas or territories, and the numbers of confirmed cases and deaths continue to rise [22,23]. Therefore, under the current public health emergency, early diagnosis is essential to control the outbreak and to select appropriate treatment. At present, RT-PCR is widely applied for clinical diagnosis [19,20,24,25]. However, there are various drawbacks to this methodology, such as low sensitivity, the occurrence of false negatives, and virus loading in oral swabs [26]. As a result of time constraints and clinical needs, the quality of the commercial kits has not been further verified and evaluated using a large number of clinical samples. The RT-PCR assay is also laborious to perform, time-consuming and requires specialized equipment. To overcome these problems, a more rapid, convenient and less complex assay is needed.

In conclusion, we developed an RT-LAMP assay for SARS-CoV-2 detection that demonstrated high diagnostic sensitivity and specificity among clinical samples. The RT-LAMP assay is a powerful tool for SARS-CoV-2 identification not only in experimental laboratories, but also in hospitals as it does not require complex equipment. It may prove useful in monitoring suspected patients, close contacts and high-risk groups.

Transparency declaration

All authors disclose no conflicts of interest.

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Contribution of authors

JY and DL designed the experiments and edited the manuscript. CY, JC, BD, GX, SL and WZ performed and developed the RT-LAMP. HY, LH, LC, LZ, YS, NL, HZ, YF, SL and QZ performed the experiments. CY and JC wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2020.04.001.

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