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Research Article

A Rapid and High-Sensitive Real-Time Reverse Transcription-Polymerase Chain Reaction Assay Used for the Detection of Severe Acute Respiratory Syndrome Coronavirus 2

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a public health emergency of international concern. Real-time reverse transcription-polymerase chain reaction (RT-PCR) is widely used as the gold standard method for the diagnosis of SARS-CoV-2 infection. However, the reliability of current real-time RT-PCR assays is questioned due to some false-negative reports. In this study, we improved the real-time RT-PCR method based on three target regions (ORF1ab, E, and N) of SARS-CoV-2. Results showed that real-time RT-PCR assays herein could complete detection within one hour after viral RNA preparation and had high sensitivity down to 5 copies of viral RNA. In addition, six clinical specimens were detected to evaluate the availability of this method. Among them, four samples were 3-plex SARS-CoV-2 positive and two were negative by real-time RT-PCR. The sensitivity was 100% (4/4), and specificity was 100% (2/2). These results demonstrate that we develop a rapid and high-sensitive real-time RT-PCR method for SARS-CoV-2 detection, which will be a powerful tool for COVID-19 identification and for monitoring suspected patients.

Keywords: COVID-19, SARS-CoV-2, RT-PCR, Diagnostic, Coronavirus

Introduction

The coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has spread around the world and has become a public health emergency of international concern [1, 2]. To date (20 August 2020), up to 22,213,869 COVID-19 cases have been confirmed, including more than 781,677 deaths [3]. In the absence of vaccines and antivirals, early diagnosis and quarantine are effective measures to cure patients and control the spread of COVID-19. The viral nucleic acid detection by real-time reverse transcription-polymerase chain reaction (RT-PCR) based on the

TaqMan Probe assay is widely employed as the gold standard method for the diagnosis of SARS-CoV-2 infection [4, 5]. Advantages of real-time RT-PCR assays are high-throughput and sensitive, and even to quantify viral loads in patients based on a standard curve for monitoring disease progression [6]. Since the global outbreak of COVID-19, real-time RT-PCR assays based on various prime/probe sets are quickly developed for SARS-CoV-2 detection [7-10]. However, their reliability is questioned due to the presence of false-negative results in some patients and positive results in some confirmed cases after recovery [11-14]. Thus, it is very important to further optimize and improve current RT-PCR assays for better screening suspected patients and preventing COVID-19 spread.

SARS-CoV-2 is an enveloped positive-sense single-stranded RNA (+ ssRNA) virus [1, 7]. Phylogenetic analyses reveal that SARS-CoV-2 belongs to β-coronavirus and its nucleotide sequence is close to bat-derived SARS-like coronaviruses [15, 16]. In order to distinguish from other high-pathogenic viruses, avoid viral mutation, and even detect the low viral load, SARS-CoV-2 is usually identified by multiple RT-PCR targeting different viral regions [4]. In this study, we optimized the real-time RT-PCR assay using four primer/probe sets targeting the open reading frame 1ab (ORF1ab), envelope protein (E), and nucleocapsid protein (N) regions of SARS-CoV-2 to improve COVID-19 detection. We also evaluated the sensitivity of this method. In addition, six clinical specimens were analyzed for the availability of this method.

Experimental

Preparation of the artificial SARS-CoV-2 gene

The synthesized sequence of the *ORF1ab*, *E*, and *N* gene of SARS-CoV-2 (BIOLIGO, Shanghai, China) were used to optimize real-time RT-PCR test. The SARS-CoV-2 RNA transcribed *in vitro* Reference Material (Shanghai Institute of Measurement and Testing Technology, Shanghai, China) containing *ORF1ab* (13321-15540, GenBank No. MT027064.1), the full-length *E* gene and *N* gene of SARS-CoV-2 quantitated by digital PCR were used to evaluate the sensitivity of real-time RT-PCR.

RNA extraction

Total RNA was extracted from the inactivated clinical specimens from Shanghai Center Clinical Laboratory (Shanghai, China) using the TaKaRa MiniBEST Viral RNA/DNA Extraction Kit Ver.5.0 (Takara, Japan) following manufacturer's instructions. The extracted RNA was stored at -80 °C.

Primers and probes

Four high-specific primer/probe sets targeting the ORF1ab, E, and N regions of SARS-CoV-2 issued by China Center for Disease Control and Prevention (CDC) [7] and Corman et al. [8] were synthesized by BIOLIGO (Shanghai, China). Target 1 (*ORF1ab*), forward: 5'-CCCTGTGGG TTTTACACTTAA-3', reverse: 5'-ACGATTG TGCATCAGCTGA-3', probe: 5'-FAM-CC GTCTGCGGTATGTGGAAAGGTTATGG-BQ1-3'; Target 2 (*E*), forward: 5'-ACAGGTACGTTAATAGTT AATAGCGT-3', reverse: 5'-ATATTGCAGCAGTACG CACACA-3', probe: 5'-FAM-ACACTAGCCATC CTTACTGCGCTTCG-BQ1-3'; Target 3 (*N1*), forward: 5'-GGGGAACTTCTCCTGCTAGAAT-3', reverse: 5'-CAGACATTTTGCTCTCAAGCTG-3', probe: 5'-FAM-TTGCTGCTGCTTGACAGATT-BQ1-3'; Target 4 (*N2*), forward: 5'-CACATTG GCACCCGCAATC-3', reverse: 5'-GAGGAACGA GAAGAGGCTTG-3', probe: 5'-FAM-ACTTCCT CAAGGAACAACATTGCCA-BQ1-3'.

Real-time RT-PCR assay

Real-time RT-PCR assay was developed using the One Step PrimerScipt^{TM} RT-PCR kit (Takara, Japan). A 25 μL reaction mixture contained 12.5 μL of 2X PCR Buffer, 0.5 μL of reverse transcriptase, 0.5 μL of *Taq* DNA Polymerase, 200 nM of probes, 1 μL of primers, and 5 μL of RNA. Real-time RT-PCR was performed using Stratagene Mx3000P (Agilent, Palo Alto CA, USA). The optimal reaction procedure was 42 °C for 5 min, 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s.

Statistical analysis

Data were presented as the mean ± standard deviation (SD). The Student's t-test was used in quantitative data analysis, and $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed with SPSS 20.0 software (SPSS, Inc., Chicago IL, USA).

Results and Discussion

Optimizing the real-time RT-PCR assay

Many commercial nucleic acid detection kits based on real-time RT-PCR assays have been widely used for SARS-CoV-2 detection, but there are differences in their detection capability of weak positive samples [17, 18]. Currently, time-consuming and insensitive methods are not able to meet the requirement of large-scale molecular diagnosis of suspected patients and asymptomatic patients. Thus, to shorten the viral detection, we first performed real-time RT-PCR assays at three different thermal cycling time. As shown in Fig. 1, the highest amplification of *ORF1ab*, *E*, and *N2* at 400 nM of primers occurred in 35 s per thermal cycle, and *N1* occurred in 25 s. Due to a multiple test, the optimal time per thermal cycle was determined at 35 s, meaning the whole real-time RT-PCR assay spent one hour completing detection.

In order to increase the amplification products by real-time RT-PCR, we performed the reaction at different primer concentration from 200 nM to 400 nM. As presented in Fig. 2, the highest amplification of *ORF1ab* occurred at 400 nM of primers, and the other three genes had no significant differences in the range of 200 to 400 nM. Therefore, the optimal primer concentration of *ORF1ab* was 400 nM, and others adopted in the subsequent studies were 200 nM.

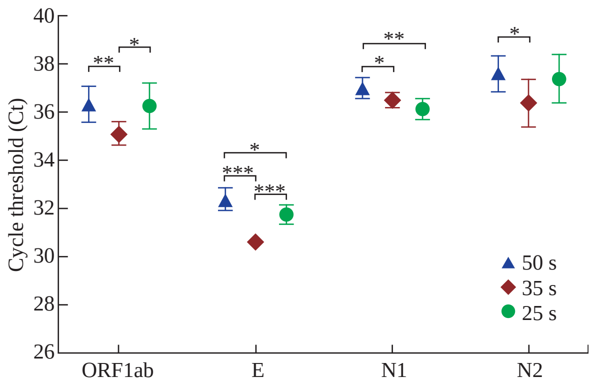

Fig. 1 Effect of thermal cycling time on real-time RT-PCR assays. Concentration of primers was 400 nM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

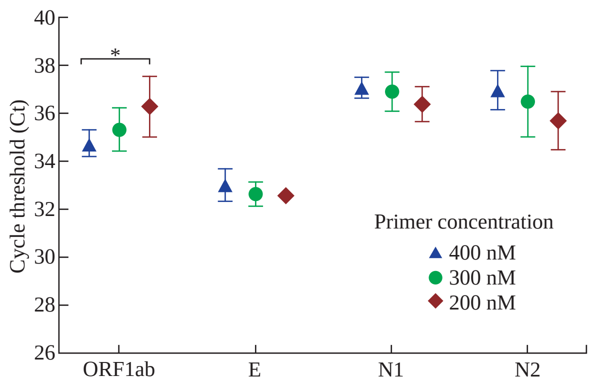

Fig. 2 Effect of primer concentration on real-time RT-PCR assays. $^{*}P < 0.05$.

Sensitivity of the real-time RT-PCR assay

The performance of the aforementioned real-time RT-PCR assay was evaluated using ten-fold serial dilutions of the viral RNA ranging from 5×10^4 copies to 5 copies. As illustrated in Fig. 3, the cycle threshold (Ct) of real-time RT-PCR was linearly linked to the number of observed viral RNA, and regression analysis produced a determination coefficient (R^2) of 0.941~0.992. The World Health Organization (WHO) posts various high-specific primer/probe sets for SARS-CoV-2 detection developed at different institutions [4]. However, assays among these primer/probe sets have different ability to detect SARS-CoV-2 [19, 20]. Chantal et al. [19] found that the most sensitive primer/probe sets in the same RT-PCR reagents and conditions are Corman E (Charité-Universitätsmedizin Berlin Institute of Virology), HKU-ORF1 (Hong Kong University), HKU-N (Hong Kong University), CCDC-N (China CDC), 2019-nCoV_N1 (United States CDC), and 2019-nCoV_N3 (US CDC), which could partially detect SARS-CoV-2 at 1 (25%) and 10 (25-50%) copies of viral RNA. Different from reaction conditions described by Chantal et al. [19], four primer/probe sets in this study also represented different amplification efficiency. The reportable range of *ORF1ab* was $5\sim5\times10^4$ copies (Fig. 3(a)), and the limit of detection (LoD) of real-time RT-PCR using *ORF1ab* analyzed with twelve replicates was 5 copies of viral RNA (12/12) (Fig. 4(a)). The *E* assay also exhibited high sensitivity (Fig. 3(b)), but it was less sensitive than *ORF1ab* (Fig. 4(b)). In fact, LoD of real-time RT-PCR using *E* primer/probe set was more than 5 copies (5/12). Compared to the *ORF1ab* and *E* assays, the *N* assay was not reliable at low SARS-CoV-2 amounts. Among two N gene assays, the *N1* primer/probe set was more sensitive than *N2* set (Fig. 3(c) and 3(d)). To further confirm the improved real-time RT-PCR method, we utilized three primer/probe (*ORF1ab*, *E* and *N1*) sets in the subsequent studies.

Clinical sample detection

Six clinical specimens were detected using the optimal real-time RT-PCR assay based on *ORF1ab*, *E*, and *N1* primer/probe sets. Among them, four 3-plex positive samples were confirmed to be infected with SARS-CoV-2, while two were negative (Table 1). The sensitivity was 100% (4/4), and specificity was 100% (2/2). In addition, the results also showed that assays using *ORF1ab* or *E* primer/probe set performed better

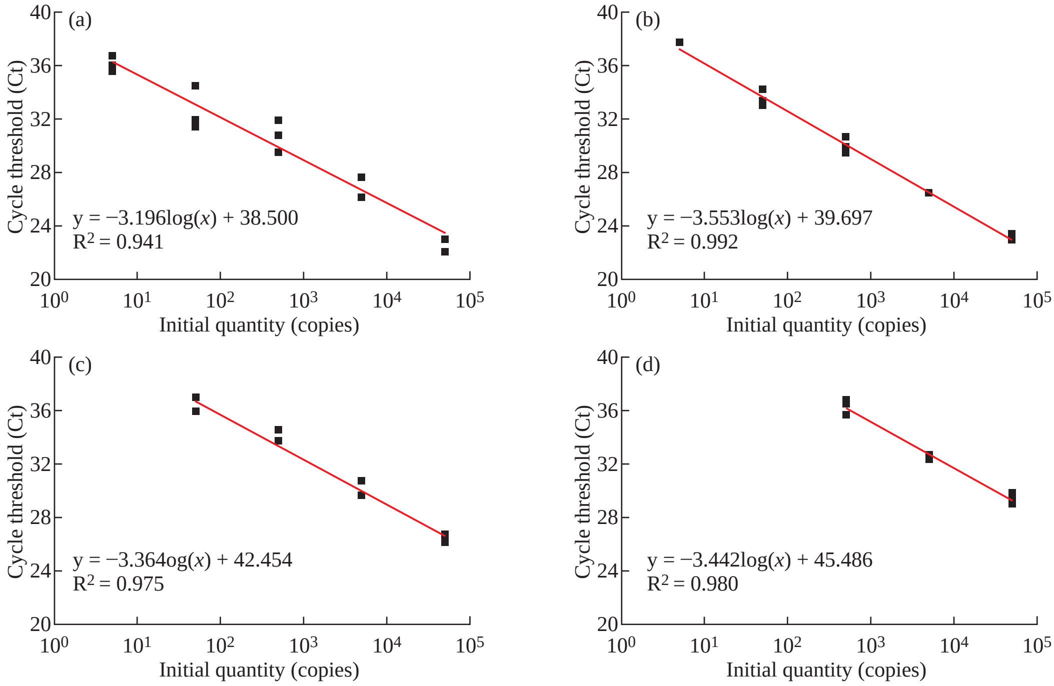

Fig. 3 Sensitivity analysis of real-time RT-PCR. The reportable range of real-time RT-PCR using **(a)** *ORF1ab*, **(b)** *E*, **(c)** *N1*, and **(d)** *N2* primer/probe sets were determined. Data were representative of three independent experiments.

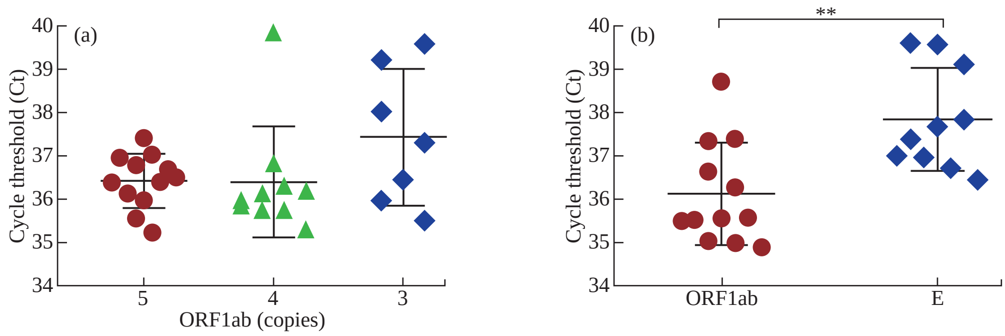

Fig. 4 **(a)** The limit of detection (LoD) of real-time RT-PCR based on *ORF1ab* primer/probe set. **(b)** Analysis of real-time RT-PCR using *ORF1ab* and *E* primer/probe sets to detect 9 copies of viral RNA. Data were analyzed with twelve replicates. $^{**}P < 0.01$.

Table 1 Clinical samples detected by real-time RT-PCR

Sample	Ct Value			Results
	ORF1ab	*E*	*N1*	
1	-	-	-	-
2	30.20	31.71	35.60	+
3	-	-	-	-
4	28.87	29.94	33.21	+
5	28.16	28.79	32.87	+
6	35.31	33.95	38.84	+

-: Negative; +: Positive.

than those using *N1* set (Table 1). Similar to most commercially available and laboratory-developed assays, we recommend that a 3-plex or 2-plex positive outcome is defined as SARS-CoV-2 positive, and not detected test result is negative. As for single-plex result, it is regarded as inconclusive and subsequently retested for the confirmation. Of course, a large scale of clinical study is necessary to prove the accuracy of this improved real-time RT-PCR method.

Conclusions

In this study, we have developed a rapid and high-sensitive real-time RT-PCR method based on the *ORF1ab* (China CDC), *N1* (China CDC), and Corman *E* primer/probe sets to improve the current SARS-CoV-2 detection. This real-time RT-PCR assay can rapidly complete detection within one hour after viral RNA preparation. Moreover, the optimal multiplexed real-time RT-PCR assay exhibits high sensitivity down to 5 copies of viral RNA. In addition, our findings indicate that *ORF1ab* and *E* assays have a higher analytical sensitivity than *N* assays, which means that *N* assays are not a reliable confirmatory assay at low SARS-CoV-2 amounts.

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Conflict of Interests

The authors declare that no completing interest exists.

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