Real-time reverse transcription-polymerase chain reaction assay panel for the detection of severe acute respiratory syndrome coronavirus 2 and its variants

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Abstract
Background: With the ongoing worldwide coronavirus disease 2019 (COVID-19) pandemic, an increasing number of viral variants are being identified, which poses a challenge for nucleic acid-based diagnostic tests. Rapid tests, such as real-time reverse transcription-polymerase chain reaction (rRT-PCR), play an important role in monitoring COVID-19 infection and controlling its spread. However, the changes in the genotypes of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants may result in decreased sensitivity of the rRT-PCR assay and it is necessary to monitor the mutations in primers and probes of SARS-CoV-2 detection over time.

Methods: We developed two rRT-PCR assays to detect the RNA-dependent RNA polymerase (RdRp) and nucleocapsid (N) genes of SARS-CoV-2. We evaluated these assays together with our previously published assays targeting the ORF1ab and N genes for the detection and confirmation of SARS-CoV-2 and its variants of concern (VOCs). In addition, we also developed two rRT-PCR assays (S484K and S501Y) targeting the spike gene, which when combined with the open reading frames (ORF)1ab assay, respectively, to form duplex rRT-PCR assays, were able to detect SARS-CoV-2 VOCs (lineages B.1.351 and B.1.1.7).

Results: Using a SARS-CoV-2 stock with predetermined genomic copies as a standard, the detection limit of both assays targeting RdRp and N was five copies/reaction. Furthermore, no cross-reactions with six other human CoVs (229E, OC43, NL63, HKU1, severe acute respiratory syndrome coronavirus and Middle East respiratory syndrome coronavirus) were observed using these assays. In addition, the S484K and S501Y assays were combined with the ORF1ab assay, respectively.

Conclusions: Four rRT-PCR assays (RdRp, N, S484K, and S501Y) were used to detect SARS-CoV-2 variants, and these assays were shown to be effective in screening for multiple virus strains.

Keywords: COVID-19; SARS-CoV-2; RT-PCR assay; Variants of concern; RNA polymerase; Nucleocapsid; SARS-CoV-2 B.1.351; SARS-CoV-2 B.1.1.7; SARS-CoV-2 20A S484K variant

Introduction

After the novel coronavirus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was identified in patients with pneumonia in December 2019,[1-3] the coronavirus disease 2019 (COVID-19) pandemic became a global phenomenon. As of March 30, 2021, 129,215,179 confirmed cases of COVID-19, including 2,820,098 deaths from 223 countries, areas, or territories, have been reported to the World Health Organization (WHO).[4] With the continuous monitoring of SARS-CoV-2 by researchers globally, new variants have been identified with mutations in key regions, such as the viral receptor-binding domain. The emergence of these variants may result in greater difficulty in controlling COVID-19.

On February 25, 2021, the WHO provided working definitions for SARS-CoV-2 variants of interest (VOIs) and variants of concern (VOCs), with the threshold for determining VOCs being greater than that for VOIs, and recommended that the greatest attention and resources should be directed at the variants with the highest public health implications. SARS-CoV-2 VOCs are associated with (i) increased transmissibility or detrimental changes in COVID-19 epidemiology, (ii) increased virulence or change in clinical disease presentation, or (iii) decreased...
effectiveness of public health and social measures and available diagnostics, vaccines, and therapeutics. At present, there are six SARS-CoV-2 VOIs and three VOCs assessed by the WHO, including lineages B.1.1.7, B.1.351, and B.1.1.28.1.

High-frequency nucleotide substitutions in the primer or probe binding regions of emerging VOIs and VOCs may result in decreased sensitivity of SARS-CoV-2 detection techniques. Some primer and probe sets have already shown nucleotide mismatches; hence, it is necessary to develop new, sensitive, and specific real-time reverse transcription-polymerase chain reaction (rRT-PCR) assays to support the public health response to SARS-CoV-2 and COVID-19. In this study, we report the development of rRT-PCR assays (RNA-dependent RNA polymerase [RdRp], nucleocapsid [N], S484K, and S501Y) to detect SARS-CoV-2 variants. We provide detailed technical data and a comprehensive comparison of the results with the performance of our published ORF1ab and N assays using multiple viral strains.

Methods

Viruses

SARS-CoV-2 strains (Pango lineage: B, B.1.351, and B.1.1.7) were isolated in our laboratory from clinical specimens obtained from SARS-CoV-2 positive patients. Human coronavirus-229E (229E-CoV), -OC43 (OC43-CoV), -NL63 (NL63-CoV), and -HKU1 (HKU1-CoV) strains were stored in our laboratory. Severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle Eastern respiratory syndrome coronavirus (MERS-CoV) infectious complementary DNA (cDNA) clones were provided by Dr Jin-Cun Zhao (Guangzhou Medical University, China).

RNA extraction

Viral RNA including SARS-CoV-2 (Pango lineage: B, B.1.351, and B.1.1.7), human coronavirus-229E (229E-CoV), -OC43 (OC43-CoV), -NL63 (NL63-CoV), and -HKU1 (HKU1-CoV) were extracted from 140 specimens obtained from SARS-CoV-2 positive patients. Approximately 60 μL of the total nucleic acid eluate was recovered into nuclease-free tubes and either tested immediately or stored at −70°C until further use.

rRT-PCR assay

The rRT-PCR assay was performed using the AgPath-IDTM One-Step RT-PCR Kit (Applied Biosystems). Each 25 μL of reaction mixture contained 12.5 μL of 2× RT-PCR buffer, 1 μL of RT-PCR enzyme mix, 5 μL of RNA, and 1 μL of synthesized probe.

Table 1: SARS-CoV-2 rRT-PCR assays primer/probe sequences.

| Target | Gene | Primer | Sequence (5’–3’) | Genome location* |
|--------|------|--------|------------------|------------------|
| ORF1ab | nsp10-11 | Forward | CCGTGTTGGTTTTACACTTAA | 13342–13362 |
|        |      | Reverse | ACGATTGTGGCATCGTGTA | 13442–13460 |
|        |      | Probe   | FAM-CCGGTGTCGGAATGTGGAAAGTTATGG-BHQ1 | 13377–13404 |
|        | N    | Forward | GGGGAACCTCTCCTGCTAGAT | 28881–28902 |
|        |      | Reverse | CAGGATTTGCCGTCTCAAGCTG | 28938–28979 |
|        |      | Probe   | FAM-CTTGGCTGCTGGATGACAGATT-BHQ1 | 28934–28953 |
|        | N9   | Forward | TTTGGTGGAAACCTCAGATTC | 28322–28341 |
|        |      | Reverse | TTGCAATGTGATGAGGACG | 28436–28455 |
|        |      | Probe   | FAM-CAGTAAACCGAGAAGCGAGTGG-BHQ1 | 28348–28374 |
| RdRp   | RdRp | Forward | CACGTTGGCAACCTCAGAGGA | 15470–15489 |
|        |      | Reverse | CCGTGACACCTGACAAATG | 15525–15544 |
|        |      | Probe   | FAM-ATGCCACACACTGTTATGCTAATAG-BHQ1 | 15491–15517 |

*The location on the reference genome, accession ID: EPI ISL_402119, EPI ISL_402120. BHQ1: Black hole quencher 1; N: Nucleocapsid; ORF: Open reading frames; RdRp: RNA-dependent RNA polymerase; rRT-PCR: Real-time reverse transcription-polymerase chain reaction; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.
Table 2: SARS-CoV-2 rRT-PCR assays limits of detection (viral RNA copies/reaction).

| Predicted No. of viral copies/reaction | ORF1ab | N | RdRp | N9 |
|--------------------------------------|--------|---|------|----|
| 20                                   | 24/24 (100.0) | 24/24 (100.0) | 24/24 (100.0) | 24/24 (100.0) |
| 10                                   | 24/24 (100.0) | 24/24 (100.0) | 24/24 (100.0) | 24/24 (100.0) |
| 5                                    | 24/24 (100.0)* | 23/24 (95.8) | 24/24 (100.0) | 24/24 (100.0)* |
| 2.5                                  | 21/24 (87.5) | 21/24 (87.5) | 20/24 (83.3) | 20/24 (83.3) |
| 1.25                                 | 18/24 (75.0) | 14/24 (58.3) | 16/24 (66.7) | 17/24 (70.8) |

*Limits of detection at which 100% of replicates were positive. ORF: Open reading frames; RdRp: RNA-dependent RNA polymerase; rRT-PCR: Real-time reverse transcription-polymerase chain reaction; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

Figure 1: (A) Relative positions of amplicon targets on SARS-CoV-2 genome. Numbers below amplicons are genome positions (accession ID: EPI_ISL_402120). Partial alignments are shown on (B) nsp10-nsp11 gene, (C) N gene, and (D) RdRp gene. Boxes indicate regions targeted by primers and probes designed in this study. E: Envelope; M: Membrane; N: Nucleocapsid; ORF: Open reading frames; RdRp: RNA-dependent RNA polymerase; S: Spike; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.
400 nmol/L each of forward and reverse primers, and 200 nmol/L of probe [Table 1]. Thermal cycling was performed at 45°C for 10 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each run included one SARS-CoV-2 genomic template control and at least two negative or mock controls (for extraction and PCR amplification). Assay specificity was determined using high-titer virus stock and infectious cDNA clones from our laboratory. Identities and viral RNA concentrations were validated by virus-specific RT-PCR before the experiment. The limit of detection was independently assessed using SARS-CoV-2 stock with predetermined genomic copies. The calibration curve for the genomic copy number was obtained using the Mx3005P Real-Time PCR System (Agilent Technologies, La Jolla, CA, USA).

**S484K and S501Y rRT-PCR assays**

The primer and probe sets for the S484K and S501Y assays are listed in Table 2. The assays were developed to target the E484K and N501Y mutations in the S gene of the B.1.351 and B.1.1.7 lineage viruses, respectively. These assays were then combined with ORF1ab to form duplex rRT-PCR assays for the preliminary screening of B.1.351 and B.1.1.7 lineage infections. The PCR mix and thermal cycling parameters for the two duplex rRT-PCR assays are as stated above in the rRT-PCR assay subsection of the “Methods” section.

**Results**

**Analysis of primer and probe signatures**

As noted above, we downloaded five SARS-CoV-2 sequences from global initiative on sharing all influenza data (https://www.gisaid.org/) that have recently garnered much attention, including three VOCs and two VOIs. These sequences were aligned to the original strain (HCoV-19/Wuhan/IVDC-HB-04/2020), and the alignment was used for assay design. Two assays (RdRp and N9) were selected based on how well they matched with the SARS-CoV-2 genome.
CoV-2 genome. The positions of the amplicons for the two assays, as well as our previously published assays (ORF1ab and N) relative to the SARS-CoV-2 genome, are shown in Figure 1A. The alignments confirmed how well the selected primers matched all sequences for the three assays (ORF1ab, RdRp, and N9). However, there were six nucleotide mismatches in the N forward primer, which predicted assay failure. Alignments of SARS-CoV-2 showing the primer and probe binding domains are presented in Figure 1B–D.

Assay sensitivity based on SARS-CoV-2 strain

Using SARS-CoV-2 viral RNA with a known copy number, we found that the four assays showed a dynamic range of approximately seven orders of magnitude ($5 \times 10^7$ copies/reaction). The amplification efficiency of the four assays ranged from 98.5% to 103.5%, $R^2 > 0.999$, in each assay [Figure 2]. To accurately determine the detection limits of these assays, serial 2-fold dilutions of quantified viral RNA (1.2520 copies/reaction) were also tested with each assay in 24-fold replicates. The lowest detection limit was defined as the dilution at which all replicates were positive for each assay. The minimum detection limit was five copies/reaction for the ORF1ab, RdRp, and N9 assays and ten copies/reaction for the N gene assay [Table 2].

Assay specificity based on hCoV RNA

The rRT-PCR assays were performed with RNA from six human coronaviruses, including four laboratory strains (229E-CoV, OC43-CoV, NL63-CoV, and HUK1-CoV) and two infectious cDNA clones (SARS-CoV and MERS-CoV). No cross-reaction was observed with any of the primer and probe sets for the ORF1ab, N, RdRp, and N9 assays.

Duplex rRT-PCR assays to detect viruses from the B.1.351 and B.1.1.7 lineages

The S484K and ORF1ab duplex rRT-PCR assays were used to detect B (original strain [HCoV-19/Wuhan/IVDC-HB-04/2020]) and B.1.351 lineage viruses, respectively. The results showed that both assays could amplify and detect the B.1.351 lineage viruses; however, only the ORF1ab assay detected the B lineage virus. Similar results were obtained using the S501Y and ORF1ab duplex rRT-PCR assays for detecting B and B.1.1.7 lineage viruses, respectively [Figure 3]. Our preliminary results suggested that the S501Y assay is as sensitive as the ORF1ab assay and the S484K assay is slightly less sensitive than the ORF1ab assay in detecting viral RNA (data not shown).

Discussion

The average evolutionary rate for coronaviruses is approximately $10^{-4}$ nucleotide substitutions per site per year, with mutations arising in each replication cycle. As the COVID-19 pandemic became global, variants of SARS-CoV-2 started garnering increasing attention. At present, the WHO has designated three SARS-CoV-2 variants as VOCs and six as VOIs. The B.1.1.7 lineage, one of the most closely monitored SARS-CoV-2 VOCs, was detected in November 2020, and it quickly spread around the world. The genetic mutations identified in this variant cause many changes in the biological characteristics of SARS-CoV-2. The N501Y substitution in the S protein has been shown to increase ACE2 binding, potentially reducing susceptibility to neutralizing antibodies with the result of increased viral transmission advantage. In addition, the S gene variations of the B.1.1.7 lineage have been associated with diagnostic test failure of a commercial S gene rRT-PCR assay, which significantly hinders the
tracking and management of the spread of SARS-CoV-2. Thus, it is imperative to align rRT-PCR primer/probe sets and SARS-CoV-2 variant sequences to assess the possible shortcomings of the current testing methods and to widen their detection spectrum.

With the emergence of SARS-CoV-2 variants, it is necessary to monitor mutations in primers and probes and update them over time. In the present study, we found an increasing number of mismatches over time in the forward primer of our previously published rRT-PCR N assay that targets the SARS-CoV-2 variant genome. Therefore, we developed two new rRT-PCR assays (RdRp and N9) that target the RdRp and N genes of SARS-CoV-2, respectively, and used these new assays to detect SARS-CoV-2 VOCs. The RdRp and N9 assay panels were shown to be both sensitive and specific, similar to our previously published rRT-PCR ORF1ab assay. The primer/probe sets targeting the RdRp gene, which has one of the lowest evolutionary rates in the SARS-CoV-2 genome, is to a certain extent resistant to mismatches, and targeting the N gene should offer enhanced sensitivity because of the relative abundance of N gene mRNA produced during virus replication. RdRp and N9 were combined with the ORF1ab assay to enhance the clinical diagnosis of SARS-CoV-2. In addition, we developed S484K and S501Y assays. When combined with the ORF1ab assay to form duplex rRT-PCR assays for preliminary detection of the SARS-CoV-2 B.1.351 and B.1.1.7 lineages, these PCRs showed good detection ability. In addition, there are some other significant mutations in SARS-CoV-2 variants, such as D614G, L452R, and E484Q, which can also be considered for specific detection with rRT-PCR assay; relevant research in our laboratory is in progress.

To conclude, we developed four rRT-PCR assays (RdRp, N, S484K, and S501Y) to detect SARS-CoV-2 variants and applied them to multiple virus strains. These assays provide a material basis for the monitoring, management, and control of SARS-CoV-2 infection.

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Conflicts of interest
None.

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