Clinical Validation of a SARS-CoV-2 Real-Time Reverse Transcription PCR Assay Targeting the Nucleocapsid Gene

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Background: Detection of SARS-CoV-2 viral RNA is important for the diagnosis and management of COVID-19.

Methods: We present a clinical validation of a reverse transcription PCR (RT-PCR) assay for the SARS-CoV-2 nucleocapsid (N1) gene. Off-board lysis on an automated nucleic acid extraction system was optimized with endemic coronaviruses (OC43 and NL63). Genomic RNA and SARS-CoV-2 RNA in a recombinant viral protein coat were used as control materials and compared for recovery from nucleic acid extraction.

Results: Nucleic acid extraction showed decreased recovery of endemic Coronavirus in vitro transcribed RNA (NL63) compared with attenuated virus (OC43). SARS-CoV-2 RNA had more reliable recovery from extraction through amplification than genomic RNA. Recovery of genomic RNA was improved by combining lysis buffer with clinical matrix before adding RNA. The RT-PCR assay demonstrated 100% in silico sensitivity and specificity. The accuracy across samples was 100% (75 of 75). Precision studies showed 100% intra-run, inter-run, and inter-technologist concordance. The limit of detection was 264 copies per milliliter (estimated 5 copies per reaction; 35.56 mean threshold cycle value).

Conclusions: This SARS-CoV-2 assay demonstrates appropriate characteristics for use under an Emergency Use Authorization. Endemic coronavirus controls were useful in optimizing the extraction procedure. In the absence of live or attenuated virus, recombinant virus in a protein coat is an appropriate control specimen type for assay validation during a pandemic.

INTRODUCTION

The global pandemic of COVID-19 (1) poses a diagnostic challenge that is best addressed by molecular diagnostic techniques. The COVID-19 pathogen, SARS-CoV-2 (2), is a single-stranded RNA Betacoronavirus with a 26-kb genome. The molecular detection of SARS-CoV-2 is based on targeting the viral genes (e.g., Orf1a/b, E, S, N genes) (3–7). In the United States, the first clinical assay available for SARS-CoV-2 was developed by the CDC (3) under a US Food and Drug

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IMPACT STATEMENT

This is a clinical validation study of a molecular assay for SARS-CoV-2, the virus that causes the disease COVID-19. Laboratories providing or considering providing SARS-CoV-2 testing may find the results to be of interest. A unique aspect of this validation is the use of alternative validation materials. In this study, we used a combination of endemic coronaviruses and commercial synthetic material to examine assay performance. This validation may be useful in the current COVID-19 outbreak and in future viral outbreaks for which molecular testing is needed.

MATERIALS AND METHODS

RNA Extraction

Under a class II biosafety cabinet, 300 μL of swabbed nasopharyngeal (NP) samples inoculated into universal viral transport medium (VTM; BD [catalog No. 220529]) or spiked control material in VTM were transferred to conical tubes containing 2 mL NucliSENS® Lysis Buffer (bioMérieux) for lysis/virus inactivation (10 minutes) before extraction. Total nucleic acids from pooled or individual residual NP collections and controls were obtained using the EMAG® Nucleic Acid Extraction System (bioMérieux) with an off-board lysis protocol and the following volume parameters: 300 μL input, 50 μL magnetic silica, and 80 μL output/elution.

Control Material and Patient Specimens

Control material for endemic coronavirus strains was obtained from Exact Diagnostics (respiratory panel pooled control including Human coronavirus NL63 [in vitro transcribed RNA] and Human coronavirus OC43 [whole inactivated virus]). These strains were detected with SYBR Green reverse transcription PCR (RT-PCR), as described previously (10, 11). SARS-CoV-2 genomic RNA was from the University of Texas Medical Branch (Galveston, TX; 6.0 X 10^7 copies/μL).
Recombinant Sindbis virus containing SARS-CoV-2 RNA was obtained as a commercial control material (Accuplex™ Reference Material [catalog No. 0505-0126]; SeraCare®).

Residual deidentified patient samples from a public health laboratory with a SARS-CoV-2 FDA EUA assay included both positive (n = 5) and negative (n = 5) samples. The transport medium of these samples was presumed to be a formulation of VTM, although the exact manufacturer is unknown. Each patient sample underwent freeze-thaw cycles at least twice before our extraction and PCR. Positive patient specimens, when indicated, were diluted in VTM before nucleic acid extraction. Twenty-five residual patient NP swab specimens in VTM collected before December 2019 were tested as SARS-CoV-2-negative samples.

Real-Time PCR

Real-time RT-PCR was performed using the SARS-CoV-2 N1 (2019-nCoV_N1) and human RNase P (RP) primer/probe mixes (IDT[catalog No. 10006606]) (Supplemental Table 1) following the CDC protocol (3) using TaqPath 1-Step RT-qPCR Master Mix, CG (ThermoFisher [catalog No. A15299]) and 5 μL of extracted nucleic acid in a final reaction volume of 20 μL. Our initial evaluation of the N1 and N2 targets suggested similar performance with slightly lower threshold cycle (Ct) values for N1. On notification that the FDA was permitting single detection of N1 or N2 for the CDC EUA, we chose to pursue the N1 target. Unlike the CDC EUA protocol that uses the ABI 7500 FAST Dx, the assay was validated on an ABI 7500. The cycling conditions are as follows: 25 °C (2 minutes), 50 °C (15 minutes), and 95 °C (2 minutes), then amplification for 40 cycles (95 °C 3 seconds, 55 °C 30 seconds) with fluorescence measured at 55 °C. The NL63 and OC43 strains were detected with SYBR Green RT-PCR as described previously (10, 11). In brief, extracted RNA was random primed for first-strand cDNA synthesis (PreSeq RNA QC; ArcherDX) and then PCR amplified (KAPA SYBR FAST qPCR; Roche). The PCR program was 1 cycle at 95 °C for 5 minutes followed by 40 cycles (95 °C for 30 seconds, 49 °C for 30 seconds, 60 °C for 45 seconds).

In Silico Sensitivity (Inclusivity) and Specificity (Cross-Reactivity)

For in silico sensitivity, forward (N1-F), reverse (N1-R), and probe (N1-TaqMan) sequences were queried in the National Center for Biotechnology Information (NCBI) BLASTN search across all Betacoronaviruses (7864 sequences on the NCBI update dated March 17, 2020). For in silico specificity, forward (N1-F), reverse (N1-R), and probe (N1-TaqMan) sequences were queried in an NCBI BLASTN search against public domain nucleotide sequences. The database search parameters were as follows: (1) standard databases were used; (2) organism was specified, and the taxonomy ID was recorded (NCBI taxid); (3) program selection was selected to optimize for “highly similar sequences” (Megablast); (4) sequences with the highest E-values were selected, and the total number of aligned bases across the whole primer or probe length were used to calculate percentage of homology.

Analytic Specificity

Specificity studies were drawn from previously collected and frozen patient NP specimens positive for microorganisms other than SARS-CoV-2 and supplemented with pooled NP specimens spiked with cultured organisms (50 μL 0.5 MacFarland bacterial or fungal isolates into 250 μL NP) or QC material (HKU1, 229E, NL63, OC43, rhinovirus type 1A, and parainfluenza virus 4; ZeptoMetrix). For bacterial or fungal isolate spiking, the following isolates were grown on blood agar, chocolate agar, or Sabouraud dextrose agar.
plates overnight at 30 °C (for *Candida albicans*) or 35-37 °C (all others), 5% CO₂: *Haemophilus influenzae* ATCC 49247, *Streptococcus pneumoniae* ATCC 49619, *Streptococcus pyogenes* ATCC 19615, *C. albicans* ATCC 10231, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, and *Streptococcus salivarius* clinical isolate.

**Accuracy, Limit of Detection, and Precision Studies**

Accuracy was determined using a combination of positive clinical specimens at various dilutions (range of undiluted to 1:20 dilution; n = 36 samples derived from 5 positive patient specimens; undiluted Ct values for the 5 positive patients ranged from 25.39 to 35.56), Accuplex-encapsulated SARS-CoV-2 (n = 9), and negative clinical specimens (n = 30). Ct values >40 for N1 were considered negative and values ≤40 Ct were considered positive. Limit of detection was tested with recombinant encapsulated SARS-CoV-2 and genomic SARS-CoV-2 RNA. For precision analysis, 2 materials were used: positive sample (SeraCare SARS-CoV-2 RNA at 1040 copies/mL) and negative sample (NP). These samples were tested in triplicate within the same assay run (intra-assay precision) and were also examined as single-sample analysis across 6 different assay runs (interassay precision). Three technologists performed nucleic acid extraction, and 2 technologists performed nucleic acid amplification procedures in 4 different paired combinations throughout the 6 assay runs. The reference method was a public health laboratory performing the CDC EUA assay.

**Statistics**

Excel 2016 (Microsoft Corp.) was used for calculations. A descriptive statistic of percentage was used. Probability values (P values) were not used for hypothesis testing.

**RESULTS**

**Extraction Optimization**

Optimization of the extraction protocol was performed using a pooled respiratory panel control material (Exact Diagnostics) that included endemic coronavirus OC43 whole inactivated virus and endemic coronavirus NL63 in vitro transcribed RNA in known concentrations. Nucleic acid recoveries of endemic coronaviruses OC43 and NL63 in NP matrix, VTM, and undiluted were compared with differing input, magnetic silica, and elution volumes. Ct values were lowest when control material was directly added to lysis buffer (Supplemental Table 2, samples 1–3). In the presence of NP or VTM matrix, there was an increase in Ct values for detecting NL63 but not OC43.

**Table 1. Limit of detection with enveloped virus containing SARS-CoV-2.**

| Virus copies/mLᵃ | Virus copies/ reaction | Total replicatesᵇ | Positive replicates | Positive rate, (%) | Mean Ct | 1 SD |
|------------------|------------------------|-------------------|--------------------|--------------------|--------|------|
| 2113             | 40                     | 20                | 19                 | 95                 | 31.66  | 1.29 |
| 1057             | 20                     | 20                | 20                 | 100                | 32.21  | 0.31 |
| 528              | 10                     | 20                | 20                 | 100                | 35.61  | 0.77 |
| 264              | 5                      | 20                | 20                 | 100                | 35.56  | 0.65 |
| 132              | 2.5                    | 3                 | 2                  | 66                 | 37.39  | 2.47 |

ᵃ Recombinant Sindbis virus containing SARS-CoV-2 RNA (Accuplex™ Reference Material).
ᵇ All concentrations were first performed in triplicate; a set of 20 replicates was examined only if all triplicate samples were detected.
(Supplemental Table 2, samples 1, 4, 7). Despite a 5-log greater concentration, detection of NL63 was similar or worse than detection of OC43 when in NP or VTM matrix (Supplemental Table 2, samples 4–9). These data suggest a loss of in vitro transcribed RNA compared with whole virus when present in matrix before a lysis step.

**In Silico Sensitivity (Inclusivity) and Specificity (Cross-Reactivity)**

An in silico analysis of sensitivity (inclusivity) and specificity (cross-reactivity) for the primers and probes used for SARS-CoV-2 assay validation was performed. BLASTN search across Betacoronaviruses (7864 sequences on March 17, 2020) identified all SARS-CoV-2 genomes (100%, representative homologies for 32 isolates in Supplemental Table 3). When combining primer and probe sequences, there was no significant homology to high-priority pathogens or organisms as defined by the FDA EUA (Supplemental Table 4).

**Assayed Specificity (Cross-Reactivity)**

Analytical cross-reactivity of primers and probes was assessed in clinical specimens positive for or spiked with common respiratory pathogens or microbiota (n = 25) and negative patient specimens (n = 5) (Supplemental Table 5). All reactions were valid, and none of the specimens were amplified by the N1 target (0 of 30).

**Limit of Detection**

Recombinant virus with SARS-CoV-2 RNA was used to determine the limit of detection of the assay by a 2-fold dilution series. A preliminary dilution in triplicate was performed; when all triplicate samples were detected, then an extended replicate series of 20 samples was examined. We found a 100% positive rate at 264 copies/mL (Table 1). This was substantially lower than we were able to achieve using genomic RNA spiked into NP samples (Table 2), which consistently recovered at only 24 × 10^6 copies/mL (mean Ct = 37.07) when tested in triplicate. However, when NP was combined with lysis buffer before spiking the RNA, recovery of RNA was improved to 750 copies/mL (Table 2). The 3-fold difference in limit of detection between virus in a recombinant protein coat compared with genomic RNA suggests loss in recovery of genomic RNA or RNA quantitation differences.

**Accuracy Study**

Residual SARS-CoV-2–positive patient specimens were tested undiluted or diluted in VTM. These positive samples were diluted because of limited availability of positive samples at the time of assay development. A total of 45 positive samples were used (36 derived from positive patient samples and 9 positive commercial reagents (Accuplex)). Thirty negative samples included residual clinical specimens confirmed negative for SARS-CoV-2 (n = 5), residual clinical samples positive for respiratory pathogens (n = 21, originally tested before December 2019), microorganism isolates (n = 2), and reference quality control material (n = 2) spiked into pooled residual NP collection matrix in VTM (total of 30 negative specimens). For accuracy, all specimens were tested in a blinded manner. All 75 specimens were concordant (Table 3 and Supplemental Table 6).

**Reproducibility Studies**

All runs were performed using aliquots of the same control material. For interassay reproducibility, the positive and negative controls were run across 6 assay runs and yielded Ct values with CVs of 1.65% and 1.02% for N1 and RP targets, respectively. For the intra-assay reproducibility, the positive and negative control samples were run in triplicate within a single run. Intra-assay reproducibility yielded CVs of 1.11% and 1.10% for N1 and RP, respectively.
The imprecision for the intertechnologist precision across 4 paired technologist combinations (1 technologist for extraction and 1 technologist for RT-PCR analysis) was 1.49% and 0.62% for N1 and RP, respectively. Concordance was 100%; the CV was <2%, and the standard deviation was <0.5 Ct (Supplemental Table 7).

**CONCLUSIONS**

In this study, we developed and validated a real-time PCR molecular assay to measure RNA from the SARS-CoV-2 virus. The limit of detection of SARS-CoV-2 was 264 copies/mL for viral protein-encapsulated RNA and 750 copies/mL for genomic RNA. In silico and analytical specificity studies showed no cross-reactivity with common respiratory pathogens. The test was validated with a total of 75 accuracy samples (45 samples positive for SARS-CoV-2 and 30 samples negative for SARS-CoV-2). The samples positive for SARS-CoV-2 RNA had Ct values ranging from 25.39 to 35.56.

A major hurdle to validation was the lack of access to SARS-CoV-2 live or inactivated virus. Purified genomic RNA was available but demonstrated variable efficiency in extraction recovery. Endemic coronaviruses were used as surrogates to optimize the extraction process. In addition, combining lysis buffer with NP specimens before spiking nonenveloped RNA improved recovery probably by decreasing RNA degradation. Improved RNA stability by spiking into matrix combined with buffer has been reported previously (12). This pre-extraction, off-board lysis protocol also has an advantage of improving the safety for
laboratory testing personnel because it can be performed in a biosafety cabinet.

The challenge of limited control material or patient specimens may arise again in future infectious disease outbreaks. The quickest specimens to be available in the COVID-19 outbreak were in vitro transcribed RNA and genomic RNA. These RNAs were helpful to optimize postextraction assay characteristics, but they showed poor extraction characteristics. In future outbreaks, production and widespread distribution of viral RNA within a recombinant protein coat would improve the speed and reliability of molecular assay validation.

In summary, we validated a modified version of the CDC assay under the FDA’s EUA with optimizations in off-board lysis and the use of SARS-CoV-2 RNA in a recombinant viral protein coat. This assay may be used in high-complexity laboratories for the diagnosis of SARS-CoV-2 in a high-throughput setting.

SUPPLEMENTAL MATERIAL

Supplemental material is available at The Journal of Applied Laboratory Medicine online.

Nonstandard Abbreviations: FDA, US Food and Drug Administration; EUA, Emergency Use Authorization; NP, nasopharyngeal; VTM, viral transport medium; RT-PCR, reverse transcription PCR; Ct, threshold cycle.

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