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VIROLOGY

Improved Molecular Diagnosis of COVID-19 by the Novel, Highly Sensitive and Specific COVID-19-RdRp/Hel Real-Time Reverse Transcription-PCR Assay Validated *In Vitro* and with Clinical Specimens

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ABSTRACT On 31 December 2019, the World Health Organization was informed of a cluster of cases of pneumonia of unknown etiology in Wuhan, China. Subsequent investigations identified a novel coronavirus, now named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), from the affected patients. Highly sensitive and specific laboratory diagnostics are important for controlling the rapidly evolving SARS-CoV-2-associated coronavirus disease 2019 (COVID-19) epidemic. In this study, we developed and compared the performance of three novel real-time reverse transcription-PCR (RT-PCR) assays targeting the RNA-dependent RNA polymerase (RdRp)/helicase (Hel), spike (S), and nucleocapsid (N) genes of SARS-CoV-2 with that of the reported RdRp-P2 assay, which is used in >30 European laboratories. Among the three novel assays, the COVID-19-RdRp/Hel assay had the lowest limit of detection *in vitro* (1.8 50% tissue culture infective doses [$TCID_{50}$]/ml with genomic RNA and 11.2 RNA copies/reaction with *in vitro* RNA transcripts). Among 273 specimens from 15 patients with laboratory-confirmed COVID-19 in Hong Kong, 77 (28.2%) were positive by both the COVID-19-RdRp/Hel and RdRp-P2 assays. The COVID-19-RdRp/Hel assay was positive for an additional 42 RdRp-P2-negative specimens (119/273 [43.6%] versus 77/273 [28.2%]; $P < 0.001$), including 29/120 (24.2%) respiratory tract specimens and 13/153 (8.5%) non-respiratory tract specimens. The mean viral load of these specimens was 3.21×10^4 RNA copies/ml (range, 2.21×10^2 to 4.71×10^5 RNA copies/ml). The COVID-19-RdRp/Hel assay did not cross-react with other human-pathogenic coronaviruses and respiratory pathogens in cell culture and clinical specimens, whereas the RdRp-P2 assay cross-reacted with SARS-CoV in cell culture. The highly sensitive and specific COVID-19-RdRp/Hel assay may help to improve the laboratory diagnosis of COVID-19.

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On 31 December 2019, the World Health Organization was informed of a cluster of cases of pneumonia of unknown etiology in Wuhan, Hubei Province, China (https://www.who.int/westernpacific/emergencies/covid-19). Subsequent investigations identified a novel coronavirus that was closely related to severe acute respiratory syndrome coronavirus (SARS-CoV) from these patients (1–3). This new virus has been recently named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the Coronavirus Study Group of the International Committee on Taxonomy of Viruses (4). Most patients with SARS-CoV-2 infection, or coronavirus disease 2019 (COVID-19), present with acute onset of fever, myalgia, cough, dyspnea, and radiological evidence of ground-glass lung opacities compatible with atypical pneumonia (5–7). However, asymptomatic or mildly symptomatic cases have also been reported (2, 8–10). Initial epidemiological investigations have indicated the Huanan seafood wholesale market in Wuhan as a geographically linked source, but subsequent detailed epidemiological assessment has revealed that up to 45% of the early cases with symptom onset before 1 January 2020 were not linked to this market (5, 11). Person-to-person transmissions among close family contacts and health care workers, including those without travel history to Wuhan, have been reported (2, 7, 12, 13). Therefore, clinical features and epidemiological links to Wuhan alone are not reliable for establishing the diagnosis of COVID-19.

As evidenced by previous epidemics caused by SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), highly sensitive and specific laboratory diagnostics for COVID-19 are essential for case identification, contact tracing, animal source finding, and rationalization of infection control measures (14–16). The use of viral culture for establishing acute diagnosis is not practical, as it takes at least 3 days for SARS-CoV-2 to cause obvious cytopathic effects in selected cell lines, such as VeroE6 cells (3). Moreover, isolation of the virus requires biosafety level 3 facilities, which are not available in most health care institutions. Serum antibody and antigen detection tests have not yet been validated, and there may be cross-reactivity with SARS-CoV, which shares a high degree (~82%) of nucleotide identity with SARS-CoV-2 (17). Because of these limitations, reverse transcription-PCR (RT-PCR) remains the most useful laboratory diagnostic test for COVID-19 worldwide.

The availability of the complete genome of SARS-CoV-2 early in the epidemic facilitated the development of specific primers and standardized laboratory protocols for COVID-19 (18, 19). The protocol of the first real-time RT-PCR assays targeting the RNA-dependent RNA polymerase (RdRp), envelope (E), and nucleocapsid (N) genes of SARS-CoV-2 were published on 23 January 2020 (20). Among these assays, the RdRp assay had the highest analytical sensitivity (3.8 RNA copies/reaction at 95% detection probability) (20). In this published RdRp assay, probe 1 was a "pan Sarbeco-Probe" which would detect SARS-CoV-2, SARS-CoV, and bat SARS-related coronaviruses, whereas probe 2 (termed "RdRp-P2" assay in the present study) was reported to be specific for SARS-CoV-2 and should not detect SARS-CoV (20). Notably, these assays were designed and validated using synthetic nucleic acid technology and in the absence of available SARS-CoV-2 isolates or original patient specimens (20). The reported RdRp assays had been implemented in >30 laboratories in Europe (21). In this study, we developed novel, highly sensitive and specific real-time RT-PCR assays for COVID-19 and compared their performances with that of the established RdRp-P2 assay using both *in vitro* and patient specimens. Clinical evaluation using different types of clinical specimens from patients with laboratory-confirmed COVID-19 showed that our novel assay targeting a different region of the RdRp/Hel was significantly more sensitive and specific than the RdRp-P2 assay.

MATERIALS AND METHODS

Viruses and clinical specimens. SARS-CoV-2 was isolated from a patient with laboratory-confirmed COVID-19 in Hong Kong (22). The viral isolate was amplified by one additional passage in VeroE6 cells to make working stocks of the virus (1.8×10^7 50% tissue culture infective doses [$TCID_{50}$]/ml). For *in vitro* specificity

evaluation, archived laboratory culture isolates ($n = 17$) of other human-pathogenic coronaviruses and respiratory viruses used were obtained from the Department of Microbiology, The University of Hong Kong, as previously described (23). All experimental protocols involving live SARS-CoV-2, SARS-CoV, and MERS-CoV followed the approved standard operating procedures of the biosafety level 3 facility as previously described (24, 25). For the clinical evaluation study, a total of 273 (120 respiratory tract and 153 non-respiratory tract) clinical specimens were collected from 15 patients with laboratory-confirmed COVID-19 in Hong Kong whose nasopharyngeal aspirate/swabs, throat swabs, and/or sputum specimens tested positive for SARS-CoV-2 RNA by the RdRp2 assay (22). Additionally, the total nucleic acid extracts of 22 archived (stored at −80°C until use) nasopharyngeal aspirates/swabs and throat swabs collected from 22 adult patients who were managed at our hospitals in Hong Kong for upper and/or lower respiratory tract symptoms that tested positive for other respiratory pathogens by FilmArray RP2 (BioFire Diagnostics, Salt Lake City, UT, USA), were prepared according to the manufacturer's instructions for assessing potential cross-reactivity of the assays with other respiratory pathogens in clinical specimens. The study was approved by Institutional Review Board of The University of Hong Kong/Hospital Authority (UW 14-249).

Nucleic acid extraction. Total nucleic acid (TNA) extraction of clinical specimens and laboratory cell culture of viral isolates were performed using a NucliSENS easyMAG extraction system (bioMérieux, Marcy-l'Étoile, France) according to the manufacturer's instructions and as previously described (24). The volume of the specimens used for extraction and the elution volume depended on the specimen type and the available amount of the specimen. In general, 250 μl of each respiratory tract specimen, urine sample, rectal swab, and feces were subjected to extraction with an elution volume of 55 μl, and 100 μl of each plasma specimen was subjected to extraction with an elution volume of 25 μl. The extracts were stored at −80°C until use. The same extracted product of each specimen was used for all the RT-PCRs.

Primers and probes. Primer and probe sets targeting different gene regions (RdRp/helicase [Hel], spike [S], and N) of SARS-CoV-2 were designed and tested. The probes were predicted to specifically detect SARS-CoV-2 and had no homologies with human, other human-pathogenic coronaviruses or microbial genes on BLASTn analysis that would potentially produce false-positive test results as previously described (23). Primer and probe sets with the best amplification performance were selected.

***In vitro* RNA transcripts for making positive controls and standards.** Linearized pCR2.1-TOPO plasmid (Invitrogen, Carlsbad, CA, USA) with a T7 promoter and a cloned target region (RdRp/Hel, S, or N) of SARS-CoV-2 were used for *in vitro* RNA transcription using MEGAscript T7 transcription kit (Ambion, Austin, TX, USA) for the standards and limit of detection (LOD) as previously described (23, 26). Each linearized plasmid template was mixed with 2 μl each of ATP, GTP, CTP, and UTP, 10× reaction buffer, and enzyme mix in a standard 20-μl reaction mixture. The reaction mixture was incubated at 37°C for 16 h, followed by the addition of 1 μl of TURBO DNase, and was further incubated at 37°C for 15 min. The synthesized RNA was cleaned by RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of purified RNA was quantified by BioDrop μLITE (BioDrop, UK).

COVID-19 real-time RT-PCR assays. Real-time RT-PCR assays for SARS-CoV-2 RNA detection were performed using QuantiNova Probe RT-PCR kit (Qiagen) in a LightCycler 480 real-time PCR system (Roche, Basel, Switzerland) as previously described (26). Each 20-μl reaction mixture contained 10 μl of 2× QuantiNova probe RT-PCR master mix, 0.2 μl of QN Probe RT-Mix, 1.6 μl of each 10 μM forward and reverse primer, 0.4 μl of 10 μM probe, 1.2 μl of RNase-free water, and 5 μl of TNA as the template. The thermal cycling condition was 10 min at 45°C for reverse transcription, 5 min at 95°C for PCR initial activation, and 45 cycles of 5 s at 95°C and 30 s at 55°C. The RdRp-P2 assay was performed as previously described (20).

Confirmation of discrepant results in different COVID-19 real-time RT-PCR assays by the LightMix Modular SARS and Wuhan CoV E-gene kit with LightCycler Multiplex RNA Virus Master. Discrepant results were confirmed by additional testing with the LightMix Modular SARS and Wuhan CoV E-gene kit (TIB Molbiol, Berlin, Germany) with LightCycler Multiplex RNA Virus Master (Roche) which could detect SARS-CoV-2, SARS-CoV, and bat SARS-like coronaviruses (*Sarbecovirus*) (LOD, 10 genome equivalent copies or less per reaction) without cross-reactivity with other human-pathogenic coronaviruses according to the manufacturer's instructions. Briefly, each 20-μl reaction mixture contained 4 μl of Roche Master, 0.1 μl of reverse transcriptase (RT) enzyme, 0.5 μl of reagent mix, 10.4 μl of water, and 5 μl of TNA as the template. The thermal cycling condition was 5 min at 55°C for reverse transcription, 5 min at 95°C for denaturation, and 45 cycles of 5 s at 95°C, 15 s at 60°C, and 15 s at 72°C.

Statistical analysis. Fisher's exact test was used to compare the performance of the assays. A P value of <0.05 was considered statistically significant. All data were analyzed with GraphPad Prism software (GraphPad Software, Inc.).

RESULTS

Design of novel COVID-19 real-time RT-PCR assays targeting different gene regions of the SARS-CoV-2 genome. Three novel real-time COVID-19 RT-PCR assays targeting the RdRp/Hel, S, and N genes of SARS-CoV-2 were developed (see Table S1 in the supplemental material). To avoid cross-reactivity with human SARS-CoV, we purposely designed the probes of our assays to contain 7 to 10 nucleotide differences with those of human SARS-CoV (strains HKU-39849 and GZ50) (see Fig. S1 in the supplemental material). In comparison, the probe of the RdRp-P2 assay contained only 3 nucleotide differences with those of human SARS-CoV (strains Frankfurt-1, HKU-39849, and GZ50) (20) (Fig. S1).

TABLE 1 Test results used for the calculation of limits of detection of the COVID-19 real-time RT-PCR assays with genomic RNA for SARS-CoV-2 in culture lysates and clinical specimens[a]

Assay	Culture lysate							Clinical specimen					
	Virus quantity ($TCID_{50}$/ml)	Intra-run *Cp*			Inter-run *Cp*			RNA extract (fold dilution)	Intra-run *Cp*			Inter-run *Cp*		
		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3		Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
COVID-19-RdRp/Hel	1.8×10^{1}	34.03	33.64	33.63	33.89	33.67	33.80	10^{-4}	34.86	34.97	34.79	35.34	35.20	34.89
	1.8×10^{0}	36.90	36.43	36.41	36.94	36.61	37.25	10^{-5}	37.74	38.05	39.45	37.95	37.96	37.83
	1.8×10^{-1}	40.00	40.00	40.00	38.52	40.00	–	10^{-6}	–	40.00	–	40.00	38.55	–
	1.8×10^{-2}	–	–	–	–	–	–	10^{-7}	–	–	–	–	–	–
COVID-19-S	1.8×10^{1}	34.88	34.96	35.08	36.32	35.94	35.64	10^{-4}	37.15	37.46	36.86	37.38	37.59	37.32
	1.8×10^{0}	36.79	36.99	37.60	38.33	39.25	38.71	10^{-5}	–	40.00	–	–	40.00	40.00
	1.8×10^{-1}	40.00	40.00	40.00	40.00	–	–	10^{-6}	–	40.00	–	–	–	–
	1.8×10^{-2}	–	–	–	–	–	–	10^{-7}	–	–	–	–	–	–
COVID-19-N	1.8×10^{1}	31.88	31.73	31.67	32.72	32.61	32.85	10^{-4}	35.64	35.01	35.10	35.52	35.38	35.62
	1.8×10^{0}	34.14	34.26	34.57	35.69	35.86	35.86	10^{-5}	39.16	40.00	39.09	40.00	38.12	37.12
	1.8×10^{-1}	38.32	37.29	36.90	40.00	38.42	–	10^{-6}	–	–	40.00	–	–	–
	1.8×10^{-2}	–	–	–	–	–	–	10^{-7}	–	–	–	–	–	–
RdRp-P2	1.8×10^{1}	33.46	33.74	33.49	33.53	33.45	33.46	10^{-4}	33.63	33.31	33.65	33.68	33.34	33.62
	1.8×10^{0}	34.05	34.64	34.12	33.78	33.83	–	10^{-5}	34.15	34.00	33.95	–	–	34.01
	1.8×10^{-1}	–	–	–	–	–	–	10^{-6}	–	–	–	–	–	–
	1.8×10^{-2}	–	–	–	–	–	–	10^{-7}	–	–	–	–	–	–

[a]Abbreviations: *Cp*, cycle number at detection threshold; –, negative.

Analytical sensitivity of the novel COVID-19 real-time RT-PCR assays. To determine the analytical sensitivity of the COVID-19 assays, we first evaluated their LODs using viral genomic RNA extracted from culture lysates and clinical specimens. Serial 10-fold dilutions of SARS-CoV-2 RNA extracted from culture lysates were prepared and tested in triplicate with each corresponding assay in two independent runs. The LOD of COVID-19-RdRp/Hel, COVID-19-S, and COVID-19-N was 1.8×10^{0} $TCID_{50}$/ml, while the LOD of RdRp-P2 was 1 log unit higher (1.8×10^{1} $TCID_{50}$/ml) (Table 1). Serial 10-fold dilutions of SARS-CoV-2 RNA extracted from a laboratory-confirmed patient's nasopharyngeal aspirate were also prepared and tested in triplicate with each corresponding assay in two independent runs. The LOD of COVID-19-RdRp/Hel and COVID-19-N (10^{-5} fold dilution) was 1 log unit lower than that of COVID-19-S and RdRp-P2 (10^{-4} fold dilution) (Table 1). On the basis of these results, we then selected the COVID-19-RdRp/Hel and COVID-19-N assays for further evaluation and determined their LODs using *in vitro* viral RNA transcripts (Table 2). The LODs of the COVID-19-RdRp/Hel and COVID-19-N assays using serial dilutions of *in vitro* viral RNA transcripts as calculated with probit analysis were 11.2 RNA copies/reaction (95% confidence interval, 7.2 to 52.6 RNA copies/reaction) and 21.3 RNA copies/reaction (95% confidence interval, 11.6 to 177.0 copies/reaction), respectively.

Comparative performance of the COVID-19-RdRp/Hel and RdRp-P2 for the detection of SARS-CoV-2 RNA in different types of clinical specimens. On the basis of the lower LOD of the COVID-19-RdRp/Hel assay compared to the LOD of the

TABLE 2 Test results used for the calculation of limits of detection of COVID-19 real-time RT-PCR assays with *in vitro* RNA transcripts for SARS-CoV-2

Predicted no. of RNA copies/reaction	No. of positive test results/no. of replicates (%)	
	COVID-19-RdRp/Hel	COVID-19-N
40	8/8 (100.0)	8/8 (100.0)
20	8/8 (100.0)	7/8 (87.5)
10	8/8 (100)	7/8 (87.5)
5	3/8 (37.5)	5/8 (62.5)
2.5	2/8 (25.0)	2/8 (25.0)
0	0/8 (0.0)	0/8 (0.0)

TABLE 3 Comparison between the COVID-19-RdRp/Hel and RdRp-P2 real-time RT-PCR assays for the detection of SARS-CoV-2 RNA in different types of clinical specimens from 15 patients with laboratory-confirmed COVID-19

Specimen type[a]	No. of positive test results/no. of specimens (%)		*P* value[b]	Mean (range) viral load (RNA copies/ml) in RdRp-P2-negative but COVID-19-RdRp/Hel-positive specimens[c]
	COVID-19-RdRp/Hel	RdRp-P2		
Respiratory tract	102/120 (85.0)	73/120 (60.8)	<0.001	4.33×10^4 ($2.85 \times 10^3 - 4.71 \times 10^5$)
NPA/NPS/TS	30/34 (88.2)	22/34 (64.7)	0.043	1.74×10^4 ($2.85 \times 10^3 - 8.40 \times 10^4$)
Saliva	59/72 (81.9)	38/72 (52.8)	<0.001	5.32×10^4 ($1.74 \times 10^3 - 4.71 \times 10^5$)
Sputum	13/14 (92.9)	13/14 (92.9)	NS	NA
Non-respiratory tract	17/153 (11.1)	4/153 (2.6)	0.005	7.06×10^3 ($2.21 \times 10^2 - 1.67 \times 10^4$)
Plasma	10/87 (11.5)	0/87 (0.0)	0.001	7.86×10^3 ($2.21 \times 10^2 - 1.67 \times 10^4$)
Urine	0/33 (0.0)	0/33 (0.0)	NS	NA
Feces/rectal swabs	7/33 (21.2)	4/33 (12.1)	NS	4.38×10^3 ($1.54 \times 10^3 - 6.69 \times 10^3$)
Total	119/273 (43.6)	77/273 (28.2)	<0.001	3.21×10^4 ($2.21 \times 10^2 - 4.71 \times 10^5$)

[a]Abbreviations: NPA, nasopharyngeal aspirate; NPS, nasopharyngeal swab; TS, throat swab.
[b]NS, not significant.
[c]NA, not applicable.

COVID-19-N assay, we then evaluated the performance of COVID-19-RdRp/Hel assay in the detection of SARS-CoV-2 RNA in clinical specimens and compared it with that of the RdRp-P2 assay. A total of 120 respiratory tract (nasopharyngeal aspirates/swabs, throat swabs, saliva, and sputum specimens) and 153 non-respiratory tract specimens (plasma and urine specimens and feces/rectal swabs) were collected from 15 patients with laboratory-confirmed COVID-19 in Hong Kong (positive nasopharyngeal aspirate/swab, throat swab, or sputum specimen by the RdRp-P2 assay). The median number of specimens collected per patient was 13. The 15 patients consisted of 8 males and 7 females. Their median age was 63 years (range, 37 to 75 years). All of them had clinical features compatible with acute community-acquired atypical pneumonia and radiological evidence of ground-glass lung opacities. At the time of writing this report, 11 patients were in stable condition, 3 were in critical condition, and 1 patient had succumbed.

Among the 273 specimens collected from these 15 patients, 77 (28.2%) were positive by the RdRp-P2 assay (Table 3). The novel COVID-2019-RdRp/Hel assay was positive for all of these 77 specimens. Additionally, the COVID-2019-RdRp/Hel assay was positive for another 42 specimens (119 positive specimens of 273 total specimens [43.6%] by COVID-2019-RdRp/Hel versus 77 positive specimens of 273 total specimens [28.2%] by RdRp-P2; $P < 0.001$), including 29/120 (24.2%) respiratory tract specimens and 13/153 (8.5%) non-respiratory tract specimens that were negative by the RdRp-P2 assay. All of these 42/273 (15.4%) additional positive specimens were confirmed to be positive by the LightMix Modular SARS and Wuhan CoV E-gene kit with the LightCycler Multiplex RNA Virus Master. The mean viral load of these specimens was 3.21×10^4 RNA copies/ml (range, 2.21×10^2 to 4.71×10^5 RNA copies/ml) and was about sixfold higher in the respiratory tract specimens (4.33×10^4 RNA copies/ml) than the non-respiratory tract specimens (7.06×10^3 RNA copies/ml).

The COVID-19-RdRp/Hel assay was significantly more sensitive than the RdRp-P2 assay for the detection of SARS-CoV-2 RNA in nasopharyngeal aspirates/swabs or throat swabs ($P = 0.043$), saliva ($P < 0.001$), and plasma ($P = 0.001$) specimens. As shown in Fig. 1, the COVID-19-RdRp/Hel assay consistently detected SARS-CoV-2 RNA in these samples with a higher sensitivity than the RdRp-2 assay throughout the patients' course of illness up to day 12 (nasopharyngeal aspirates/swabs and/or throat swabs) to day 18 (saliva). The sensitivity of the two assays did not differ significantly for sputum specimens and feces/rectal swabs.

Cross-reactivity of the novel COVID-19-RdRp/Hel and COVID-19-N assays with other human-pathogenic coronaviruses and respiratory viruses. The SARS-CoV-2 genome is highly similar to that of human SARS-CoV, with an overall ~82% nucleotide identity (17). RT-PCR assays that target gene fragments that are homologous in both viruses

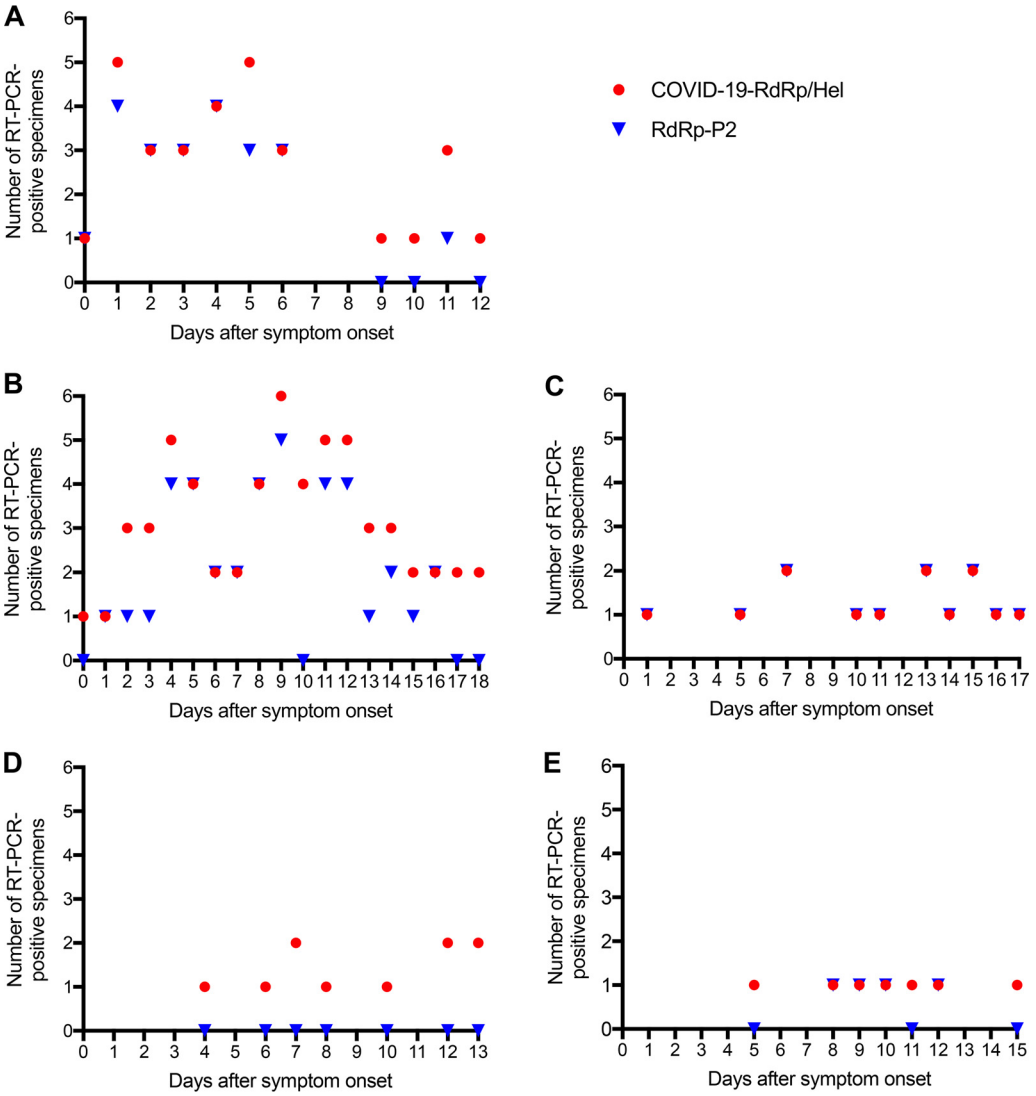

FIG 1 The number of clinical specimens that were positive for SARS-CoV-2 RNA by the COVID-19-RdRp/Hel assay or RdRp-P2 assay on different days after symptom onset from nasopharyngeal aspirates/swabs and/or throat swabs (A), saliva specimens (B), sputum specimens (C), plasma specimens (D), and feces or rectal swabs (E).

may therefore be nonspecific. To investigate whether the novel COVID-19-RdRp/Hel and COVID-19-N assays cross-react with SARS-CoV, other human-pathogenic coronaviruses, and respiratory viruses, we used the assays to test 17 culture isolates of coronaviruses (SARS-CoV, MERS-CoV, human coronavirus HCoV-OC43, HCoV-229E, and HCoV-NL63), adenovirus, human metapneumovirus, influenza A (H1N1 and H3N2) viruses, influenza B virus, influenza C virus, parainfluenza viruses types 1 to 4, rhinovirus, and respiratory syncytial virus. As shown in Table 4, no cross-reactivity with these viruses was found in either assay. Unlike what was previously reported, we found that the RdRp-P2 assay cross-reacted with SARS-CoV culture lysate (20). This cross-reactivity was consistently observed in two independent runs conducted on different days with each run having three technical replicates of each biological replicate (two biological replicates, SARS-CoV strains HKU-39849 and GZ50) and stringent compliance with the published protocol.

To investigate whether the COVID-19-RdRp/Hel assay was specific for SARS-CoV-2 in clinical specimens, we used the assay to test 22 archived nasopharyngeal aspirates/swabs and throat swabs that were positive for other respiratory pathogens by FilmArray RP2 from 22 patients with upper and/or lower respiratory tract symptoms. As shown in Table 5, none of these specimens was positive by the COVID-19-RdRp/Hel assay,

TABLE 4 Cross-reactivity between the COVID-19 real-time RT-PCR assays and other respiratory viruses in cell culture

Virus[a]	Viral titer ($TCID_{50}$/ml)[b]	Cross-reactivity[c]		
		COVID-19-RdRp/Hel	COVID-19-N	RdRp-P2
SARS-CoV	1.0×10^3	−	−	+
MERS-CoV	5.6×10^3	−	−	−
HCoV-OC43	3.2×10^3	−	−	−
HCoV-229E	5.0×10^2	−	−	−
HCoV-NL63	3.2×10^1	−	−	−
Adenovirus	1.0×10^2	−	−	−
hMPV	3.2×10^2	−	−	−
IAV (H1N1)	4.2×10^3	−	−	−
IAV (H3N2)	5.6×10^3	−	−	−
IBV	3.2×10^3	−	−	−
ICV	5.6×10^2	−	−	−
PIV1	1.0×10^2	−	−	−
PIV2	1.0×10^3	−	−	−
PIV3	1.0×10^3	−	−	−
PIV4	1.0×10^3	−	−	−
Rhinovirus	7.9×10^3	−	−	−
RSV	1.0×10^3	−	−	−

[a]Abbreviations: HCoV, human coronavirus; hMPV, human metapneumovirus; IAV, influenza A virus; IBV, influenza B virus; ICV, influenza C virus; MERS-CoV, Middle East respiratory syndrome coronavirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus; SARS-CoV, severe acute respiratory syndrome coronavirus.
[b]The same viral titers were used for all the assays. $TCID_{50}$, 50% tissue culture infective dose.
[c]+, positive; −, negative.

suggesting that the assay was specific for the detection of SARS-CoV-2 RNA in nasopharyngeal aspirates/swabs and throat swabs containing DNA/RNA of other human-pathogenic coronaviruses and respiratory pathogens.

DISCUSSION

The positive-sense, single-stranded RNA genome of SARS-CoV-2 is ~30 kb in size and encodes ~9,860 amino acids (2, 17, 18, 27). Like other betacoronaviruses, the SARS-CoV-2 genome is arranged in the order of 5′-replicase (ORF1a/b)-spike (S)-envelope (E)-membrane (M)-nucleocapsid (N)-poly(A)-3′ (17). Traditionally, the preferred targets of coronavirus RT-PCR assays included the conserved and/or abundantly expressed genes such as the structural S and N genes and the nonstructural RdRp and replicase open reading frame (ORF) 1a/b genes (16, 28). For COVID-19, the protocols of a number of RT-PCR assays used by different institutes have recently been made available online (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance). These assays target the ORF1a/b, ORF1b-nsp14, RdRp, S, E, or N gene of SARS-CoV-2, and some are nonspecific assays that would

TABLE 5 Lack of cross-reactivity between the COVID-19-RdRp/Hel assay and other respiratory pathogens in clinical specimens[a]

Organism found by FilmArray RP2[b]	No. COVID-19-RdRp/Hel-positive specimens/no. of total specimens
HCoV-OC43	0/2
HCoV-HKU1	0/1
HCoV-229E	0/1
Adenovirus	0/3
IAV	0/7
PIV	0/3
Rhinovirus/enterovirus	0/4
Mycoplasma pneumoniae	0/1
Total	0/22

[a]The clinical specimens included nasopharyngeal aspirates, nasopharyngeal swabs, and throat swabs tested by FilmArray RP2.
[b]Abbreviations: HCoV, human coronavirus; IAV, influenza A virus; PIV, parainfluenza virus.

detect SARS-CoV-2 and other related betacoronaviruses such as SARS-CoV (20, 29). Importantly, the in-use evaluation data of these assays using a large number of clinical specimens from patients with confirmed COVID-19 are lacking. In this study, we developed and evaluated three novel real-time RT-PCR assays that target different gene regions of the SARS-CoV-2 genome. We showed that the novel COVID-19-RdRp/Hel assay was highly sensitive and specific for the detection of SARS-CoV-2 RNA *in vitro* and in COVID-19 patient specimens.

Among the three assays developed in this study, the COVID-19-RdRp/Hel assay has the lowest LOD with *in vitro* viral RNA transcripts (11.2 RNA copies/reaction; 95% confidence interval; 7.2 to 52.6 RNA copies/reaction). The LOD with genomic RNA was also very low (1.80 $TCID_{50}$/ml). Importantly, the COVID-19-RdRp/Hel assay was significantly more sensitive ($P \leq 0.001$) than the established RdRp-P2 assay for the detection of SARS-CoV-2 RNA in both respiratory tract and non-respiratory tract clinical specimens. The COVID-19-RdRp/Hel assay detected SARS-CoV-2 RNA in 42/273 (15.4%) additional specimens that tested negative by the RdRp-P2 assay. These findings are clinically and epidemiologically relevant, because asymptomatic and mildly symptomatic cases of COVID-19 have been increasingly recognized, and these patients with cryptic pneumonia may serve as a potential source for propagating the epidemic (2, 8). Given the large number of patients (>60,000 cases in China at the time of writing) involved in this expanding epidemic, the additional positive specimens detected by the COVID-19-RdRp/Hel assay might translate into thousands of specimens that would otherwise be considered SARS-CoV-2 negative by the less-sensitive RdRp-P2 assay.

Regarding the different types of clinical specimens, the COVID-19-RdRp/Hel assay was significantly more sensitive than the RdRp-P2 assay for the detection of SARS-CoV-2 RNA in nasopharyngeal aspirates/swabs or throat swabs, saliva specimens, and plasma specimens. False-negative results might arise from testing nasopharyngeal aspirate/swabs or throat swabs with low viral loads in COVID-19, SARS, and MERS patients (2, 30–33). RT-PCR assays with higher sensitivity, such as the COVID-19-RdRp/Hel assay, might help to reduce the false-negative rate among these specimens which are frequently the only specimens available for establishing the diagnosis of COVID-19. We have previously shown that saliva has a high concordance rate with nasopharyngeal aspirates for the detection of influenza viral RNA and might also be a suitable specimen for diagnosing COVID-19 (22, 34). The use of the highly sensitive COVID-19-RdRp/Hel assay to test saliva specimens from suspected cases of COVID-19 might be a simple and rapid way to avoid the need of aerosol-generating procedures during collection of nasopharyngeal aspirates and suction of sputum, especially in regions most heavily affected by the ongoing COVID-19 outbreak where there are insufficient supplies of full personal protective equipment (13). SARS-CoV-2 RNAemia has been reported in a small proportion of COVID-19 patients (2, 5). However, as shown in our clinical evaluation in which the RdRp-P2 assay gave negative test results for all 10 plasma specimens that gave positive test results by the COVID-19-RdRp/Hel assay, the genuine incidence of SARS-CoV-2 RNAemia might be underestimated by less sensitive RT-PCR assays. We have previously shown that high serum viral loads in SARS patients were associated with more severe disease as evidenced by a higher incidence of oxygen desaturation, need for mechanical ventilation, hepatic dysfunction, and death (35). Thus, serial monitoring of the plasma viral load in COVID-19 patients with the highly sensitive COVID-19-RdRp/Hel assay should be considered to provide prognostic insights and facilitate treatment decisions.

The COVID-19-RdRp/Hel assay was highly specific and exhibited no cross-reactivity with other common respiratory pathogens *in vitro* and in nasopharyngeal aspirates. Interestingly, our evaluation showed that the RdRp-P2 assay cross-reacted with SARS-CoV *in vitro*, which is different from what was previously reported (20). We postulated that this might be due to the small number ($n = 3$) of nucleotide differences between the probe used in the RdRp-P2 assay with at least three strains of SARS-CoV (20). This cross-reactivity would be especially important for laboratories in areas where SARS-CoV

might reemerge and cocirculate with SARS-CoV-2, as the differences in clinical progression between SARS and COVID-19 remain incompletely understood at this stage.

The main limitation of this study was that the COVID-19-RdRp/Hel and RdRp-P2 assays were performed using different commercially available reagents, primer/probe concentrations, and cycling conditions, which made it challenging to determine the root of the difference in sensitivity. Nevertheless, our data showed that the newly established COVID-19-RdRp/Hel assay was highly sensitive and specific for the detection of SARS-CoV-2 RNA *in vitro* and in respiratory and non-respiratory tract clinical specimens. The use of this novel RT-PCR assay might be especially useful for detecting COVID-19 cases with low viral loads and when testing upper respiratory tract, saliva, and plasma specimens from patients. Development of COVID-19-RdRp/Hel into a multiplex assay which can simultaneously detect other human-pathogenic coronaviruses and respiratory pathogens may further increase its clinical utility in the future.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.4 MB.

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A U.S. provisional patent application (62/980,094) has been filed for the findings in this study (36).

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