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Development of two TaqMan real-time reverse transcription-PCR assays for the detection of severe acute respiratory syndrome coronavirus-2

Yiwei Liu [a,1], Yingying Wang [a,1], Xinming Wang [a], Yan Xiao [a], Lan Chen [a], Li Guo [a], Jianguo Li [b,**], Lili Ren [a,c,*], Jianwei Wang [a,c]

[a] National Health Commission Key Laboratory of Systems Biology of Pathogens and Christophe Mérieux Laboratory, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100730, China
[b] Institutes of Biomedical Sciences, Shanxi University, Taiyuan 030006, China
[c] Key Laboratory of Respiratory Disease Pathogenomics, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China

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ABSTRACT

The outbreak of coronavirus disease 2019 (COVID-19) in Wuhan, China, was caused by a novel coronavirus (CoV), named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The rapid detection of viral nucleic acids is critical for the early identification of infected cases. We have developed two TaqMan real-time reverse transcription-PCR assays to detect SARS-CoV-2. The designed primers target the nucleocapsid (N) and open reading frame (ORF) 1b gene regions, where the probes discriminate SARS-CoV-2 from other human and animal CoVs. The sensitivities are one genomic copy per reaction for the N gene assay and ten copies for the ORF 1b gene assay. The overall linear detection ranges are 1–10^6 and 10–10^6 copies per reaction for the N gene assay and the ORF 1b gene assay, respectively. Surveillance of 23 suspected COVID-19 patients demonstrated that SARS-CoV-2 could be detected from 100% (23/23) and 62.5% (16/23) of clinical specimens by the N gene assay and the ORF 1b gene assay, respectively. All of the samples not detected by the ORF 1b gene assay were throat swabs, indicating a lower viral load in the upper respiratory tract and the relatively lower sensitivity of the ORF 1b gene assay. The assays developed in the present study offer alternative diagnostic tests for COVID-19.

1. Introduction

In late 2019, a novel coronavirus disease 2019 (COVID-19) emerged in Wuhan, Hubei province, China [1–3]. The etiological agent was later identified as a novel coronavirus (CoV), now named severe acute respiratory syndrome CoV-2 (SARS-CoV-2) [4]. Rapid and reliable diagnostic tests of SARS-CoV-2 were needed for quick confirmation of the viral infection and for the guidance of medical interventions [5,6]. In response, several panels of TaqMan real-time reverse transcription-PCR (RT-PCR) assays for the detection of SARS-CoV-2 were quickly suggested by the World Health Organization, the Chinese Center for Disease Control and Prevention and some biotechnology companies.

The requirement for rapid and reliable diagnostics of SARS-CoV-2 is increasing to meet the demands of accurate diagnosis and to document infections [6,7]. However, most of the currently developed TaqMan real-time RT-PCR assays for the detection of SARS-CoV-2 were designed as an emergency response and their analytical performance has not been reported in detail, including the sensitivity, specificity and linear detection range of the assays. Some assays were even developed in the absence of SARS-CoV-2 isolates or patient samples [5]. Comparisons with the currently available real-time RT-PCR assays are also lacking [5].

To provide a more reliable choice for the rapid detection of SARS-CoV-2 and to comprehensively evaluate the analytical performance of the real-time RT-PCR assays, we developed a panel of two novel TaqMan real-time RT-PCR assays and evaluated their analytical performance in the detection of SARS-CoV-2.

2. Materials and methods

2.1. Nucleic acid extraction

The clinical specimens (sputum and throat swab) were taken from patients suspected of COVID-19 (one sample from each patient), and were kept in viral transport medium (VTM). All of the clinical

* Corresponding author: Chinese Academy of Medical Sciences & Peking Union Medical College, No.9 Dong Dan San Tiao, Dongcheng District, Beijing 100730, China.
** Corresponding author: Shanxi University, No. 92 Wucheng Road, Xiaodian District, Taiyuan 030006, China.
E-mail addresses: lijg@sxu.edu.cn (Jianguo Li), renliliipb@163.com (Lili Ren).
[1] These authors contributed equally to this work.

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HIGHLIGHTS

Scientific question

The rapid detection of viral nucleic acids is critical for early identification of the infected cases. We would like to develop sensitive and specific TaqMan real-time RT-PCR assays for detecting SARS-CoV-2 in clinical specimens, and analytically investigate the performance of them in detail.

Evidence before this study

The outbreak of SARS-CoV-2 caused pandemic globally. The requirement for rapid and reliable diagnostics of SARS-CoV-2 is increasing to meet the demand of accurate diagnosis and document infections. However, most of the currently developed TaqMan real time RT-PCR assays for the detection of SARS-CoV-2 were aided for emergency technology support, the analytical performance of which have not been reported in detail, including the sensitivity, specificity, linear detection range, etc. Some assays were even developed in the absence of SARS-CoV-2 isolate or patients samples.

New findings

In this study we introduced TaqMan real-time reverse transcription-PCR assays to detect SARS-CoV-2, targeting to the nucleocapsid (N) and the open reading frame 1b (ORF 1b) gene region. The sensitivities are one genomic copy per reaction for the N gene assay and 10 copies for the ORF 1b gene assay. The overall linear detection ranges are 1–10^6 and 10–10^6 copies per reaction for the N gene assay and the ORF 1b gene assay, respectively. The specificities are also discussed. All of the samples not detected by the ORF 1b gene assay were throat swabs, indicating a lower viral load in upper respiratory tract and a relative low sensitivity of the ORF 1b gene assay.

Significance of the study

We developed two TaqMan real-time RT-PCR assays permitting rapid, sensitive, and specific detection of SARS-CoV-2 in clinical specimens.

specimens were treated in a Biosafety Level 3 laboratory. A total of 200 μl VTM were added into 600 μl Trizol LS for lysis, and subsequent RNA extraction was performed by using Direct-zol™ RNA MiniPrep (ZYMO RESEARCH, CA, USA) according to the manufacturer's instructions. The extracted RNA was resolved in a volume of 50 μl DNase/RNase-Free water and either tested immediately or stored at −80 °C.

The total nucleic acids were extracted by using a NucliSens easyMAG apparatus (bioMerieux, Marcy l' Etoile, France) from respiratory samples positive for other common respiratory viruses, including human coronaviruses (NL63, OC43, 229E, and HKU1), influenza A and B, respiratory syncytial virus, parainfluenza 1 to 4, human metapneumovirus, rhinovirus, adenovirus, and bocavirus, which in the sample bank within our laborator, confirmed as reported previously [1,8].

2.2. Primers and probes

Two sets of primers and probes (targeting the nucleocapsid (N) gene and open reading frame [ORF] 1b gene, respectively) were designed based on the genome of SARS-CoV-2 (GenBank accession No. MN908947.3) by Primer Express software version 3.0 (Thermo Fisher Scientific, Waltham, MA, USA). The following set of principles were applied to the design of the primers and probes: primer length: 18–25 bp; primer Tm (melting temperature): 55–60 °C; primer G + C content: 40%–60%; probe length: 20–30 bp; probe Tm: 60–65 °C; probe G + C content: 40%–60%; lacking three or more consecutive G or C bases; lacking G at the 5′ end; and no obvious dimer or hairpin structure.

We aligned our primers and probes with other related human and animal coronaviruses (Human CoV: HKU1 (NC_006577), OC43 (KF923896), NL63 (NC_005831), and 229E (NC_002645); Bat SARS-like CoV: KT444582, KC881005, and KY417144; *Rhinolophus affinis* CoV: KF569996; Bat CoV: HKU10 (NC_018871), HKU8 (NC_010438), HKU2 (NC_009988), HKU9 (NC_009021), HKU4 (NC_009019), and HKU5 (NC_009020); SARS CoV: AY278488, AY304486; Avian infectious bronchitis virus: NC_001451; and Middle East Respiratory Syndrome CoV: NC_019843). The aligned results (Fig. 1) showed low sequence identity between the above viral genomes and the primers and probes designed in the present study.

All primers and probes were synthesized by standard phosphoramidite chemistry techniques at Qingke Biotechnology Co. Ltd. (Beijing, China). TaqMan probes were labeled with the molecule 6-carboxy-fluroscein at the 5′ end, and with the quencher Blackhole Quencher 1 at the 3′ end. Optimal concentrations of the primers and probes were determined by cross-titration of serial two-fold dilutions of each primer/probe against a constant amount of purified SARS-CoV-2 RNA. Primers and probes that exhibited the highest amplification efficiencies in the present study were selected for further evaluation (Fig. 1, Table 1).

2.3. TaqMan real-time RT-PCR assay

The TaqMan real-time RT-PCR assays were performed using TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific). Each 20 μl reaction mix contained 5 μl of 4× Fast Virus 1-Step Master Mix, 0.2 μl of 50 μM probe, 0.2 μl each of 50 μM forward and reverse primers, 12.4 μl of nuclease-free water, and 2 μl of extracted RNA. Plasmids containing the primer-targeted viral gene regions were taken as positive control, and DNase/RNase-Free water was taken as negative control. Amplifications were carried out in 96-well plates using a Bio-Rad instrument (Bio-Rad CFX96, Hercules, CA, USA). Thermocycling conditions were as follows: 15 min at 50 °C for reverse transcription, 4 min at 95 °C for predenaturation, followed by 45 cycles of 15 s at 95 °C and 45 s at 60 °C. Fluorescence measurements were taken at 60 °C during each cycle. The threshold cycle (Ct) value was determined by the point at which fluorescence exceeded a threshold limit set at the mean plus 10 standard deviations above the baseline. A result was considered positive if two or more of the SARS-CoV-2 genome targets exhibited positive results ($Ct \leq 35$). A result of $35 \leq Ct \leq 40$ was considered as a suspected case and a repeat test was performed for confirmation.

2.4. Preparation of RNA transcripts

RNA transcripts for the N gene and the ORF 1b gene of SARS-CoV-2 were prepared. Plasmids (pEasy-T1, TransGen Biotech, Beijing, China) inserted with the viral gene regions (including N and ORF 1b, respectively) were linearized by digestion with restriction enzyme BamHI, and transcribed in vitro using the RiboMAX™ Large Scale RNA Production System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The concentration of the RNA transcripts was determined using NanoDrop technology (Thermo Fisher Scientific).

3. Results

3.1. The sensitivity and reproducibility of the developed TaqMan real-time RT-PCR assays

To determine the sensitivity of the real-time RT-PCR assays, we analyzed the detection limits using ten-fold dilutions of the RNA transcripts

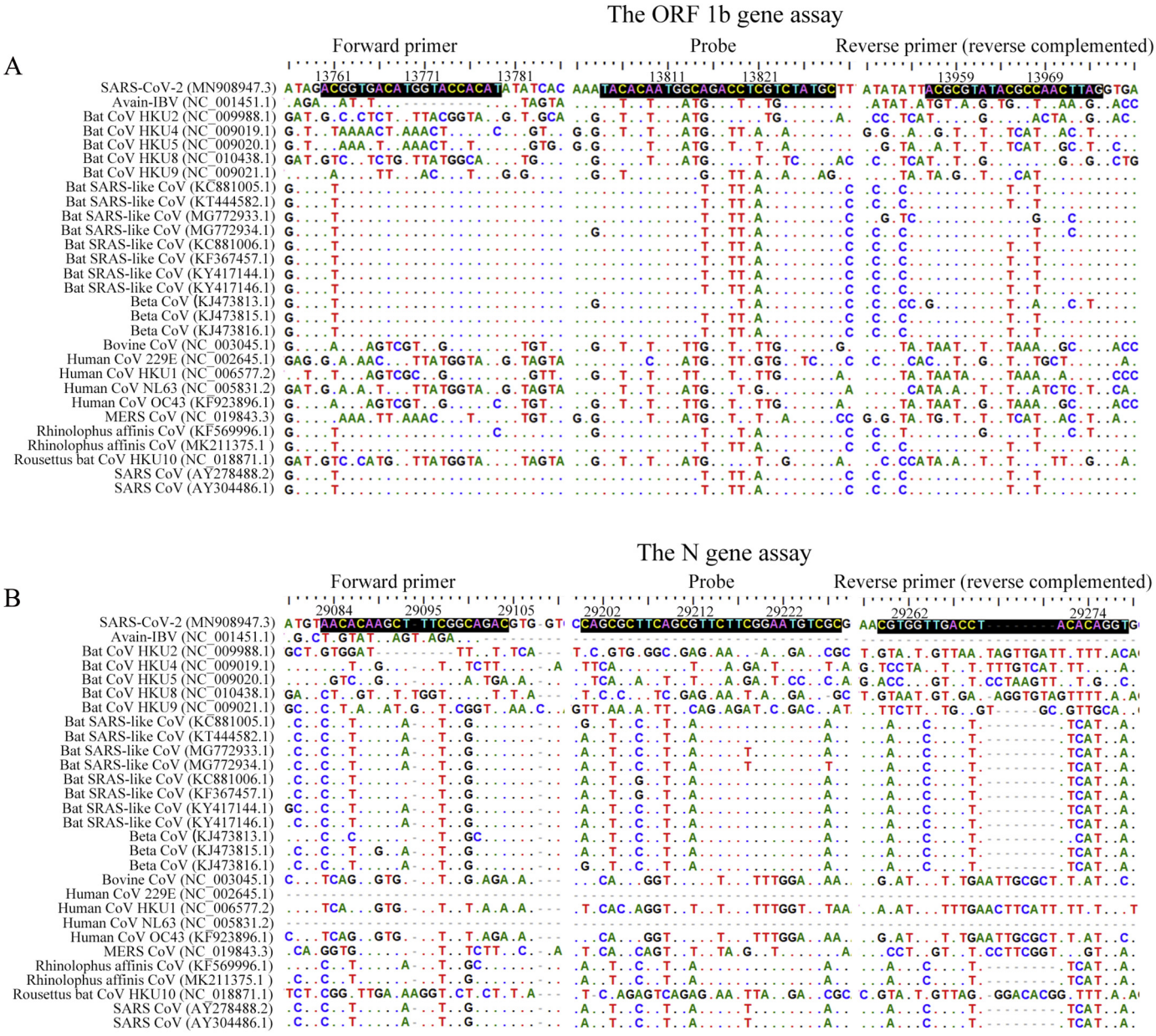

Fig. 1. Alignment of the designed primers/probes with the genomic sequences of SARS-CoV-2 and phylogenically closely-related coronaviruses. Alignment of the primers and probes for the N gene assay (A) and the ORF 1b gene assay (B) against the corresponding genomic sequences of coronaviruses. The genome of SARS-CoV-2 and other related coronaviruses were retrieved from the GenBank database and aligned using ClustalW software. The viral genomic regions targeted by primers and probes designed in the present study are displayed in inverse color along with the genomic locations of the 5′- and 3′-ends corresponding to the genome of SARS-CoV-2 (GenBank accession No. MN908947.3). Avian IBV: avian infectious bronchitis virus.

Table 1
Primers and probes used for the TaqMan real-time RT-PCR assays.

Gene	Primer/probe	Sequence (5′–3′)	Genomic location[a]	Amplicon (bp)
ORF 1b	Forward	ACGGTGACATGGTACCACAT	13,760–13,779	215
	Reverse	CTAAGTTGGCGTATACGCGT	13,975–13,956	
	Probe	TACACAATGGCAGACCTCGTC TATGC	13,804–13,829	
N	Forward	AACACAAGCTTTCGGCAGAC	29,083–29,102	195
	Reverse	ACCTGTGTAGGTCAACCACG	29,278–29,259	
	Probe	CAGCGCTTCAGCGTTCTTCGG AATGTCGC	29,200–29,228	

[a] Numbering according to a reference genome of SARS-CoV-2 (MN908947.3).

of the N and ORF 1b genes as templates. The results revealed that one copy per reaction of N RNA and ten copies per reaction of ORF 1b RNA could be detected by the N gene assay and the ORF 1b gene assay, respectively (Table 2). The Ct values increased as the N and ORF 1b RNA copies ranged from 10^6 to one copy in the reaction (Fig. 2, Table 2). Strong linear correlations ($r^2 = 0.9958$ for the N gene assay and $r^2 = 0.9963$ for the ORF 1b gene assay) were obtained between Ct values and the quantity of RNA transcripts. These results suggested that the developed assays possess high sensitivities and wide linear ranges in SARS-CoV-2 detection.

The reproducibility of the developed assays was then analyzed by assessing the intra- and inter-assay variabilities (Table 3). Reproducibility reached 100% at one copy per reaction for the N gene assay and 10 copies per reaction for the ORF 1b gene assay. The coefficients of the mean Ct

Table 2
Efficiency of the TaqMan real-time RT-PCR assays.

Gene	Template	Mean Ct values at quantified RNA copy number (copy/reaction)							Slope[a]	Efficiency[b]
		1×10^6	1×10^5	1×10^4	1×10^3	1×10^2	1×10^1	1×10^0		
N	RNA transcript alone	17.62 ± 0.52[c]	21.98 ± 1.33	25.07 ± 0.33	28.25 ± 0.45	31.00 ± 0.20	34.73 ± 1.24	37.15 ± 2.34	−3.22	104%
	RNA transcript + other viruses[d]	19.40 ± 0.19	24.40 ± 0.04	26.38 ± 0.09	28.98 ± 0.07	31.17 ± 0.28	33.17 ± 0.26	36.76 ± 0.58	−3.01	113%
ORF 1b	RNA transcript alone	18.64 ± 0.73	22.20 ± 0.26	25.73 ± 0.28	28.83 ± 0.37	31.89 ± 1.41	34.42 ± 0.28	37.22 ± 1.15	−3.08	108%
	RNA transcript + other viruses	20.36 ± 0.06	23.49 ± 0.13	26.46 ± 0.18	29.88 ± 0.12	32.91 ± 0.40	35.39 ± 2.12	37.89 ± 0.58	−2.92	118%

[a] Slope determined from the formula: Y = Y intercept − slope log10.
[b] Efficiency = [10(−1/slope)] − 1.
[c] Values shown are the mean of triplicate samples ± standard deviation.
[d] "RNA transcript" represents the in vitro transcribed RNA of the corresponding genes of SARS-CoV-2. "Other viruses" represents the pooled RNA extracted from 15 human respiratory specimens by using Trizol. "RNA transcript + other viruses" represents a 1:1 (*v*/*v*) mixture of these two components.

values within and between runs for the N gene assay were 0.20%–1.33% and 0.77%–2.45%, respectively, compared with 0.26%–4.45% and 0.49%–5.12%, respectively, for the ORF 1b gene assay. These results indicated the high reproducibility of the developed assays.

3.2. The amplification efficiencies of the assays

The amplification efficiencies of the assays were assessed through the amplification of RNA transcripts in the presence of exogenous nucleic acids from other common respiratory viruses, including influenza A and B, respiratory syncytial virus, parainfluenza virus 1–4, adenovirus, bocavirus, rhinovirus, and enterovirus. Ten-fold serial dilutions of the RNA transcripts were prepared in RNase-free water (RNA transcript alone) and the pooled RNA from 15 SARS-CoV-2-negative human respiratory specimens (RNA transcript + other viruses). The standard curve of the N gene assay had a lower efficiency in water (104%, slope = −3.22) than in the presence of RNA from other respiratory viruses (113%, slope = −3.01) (Table 2). A similar efficiency trend was observed for the ORF 1b

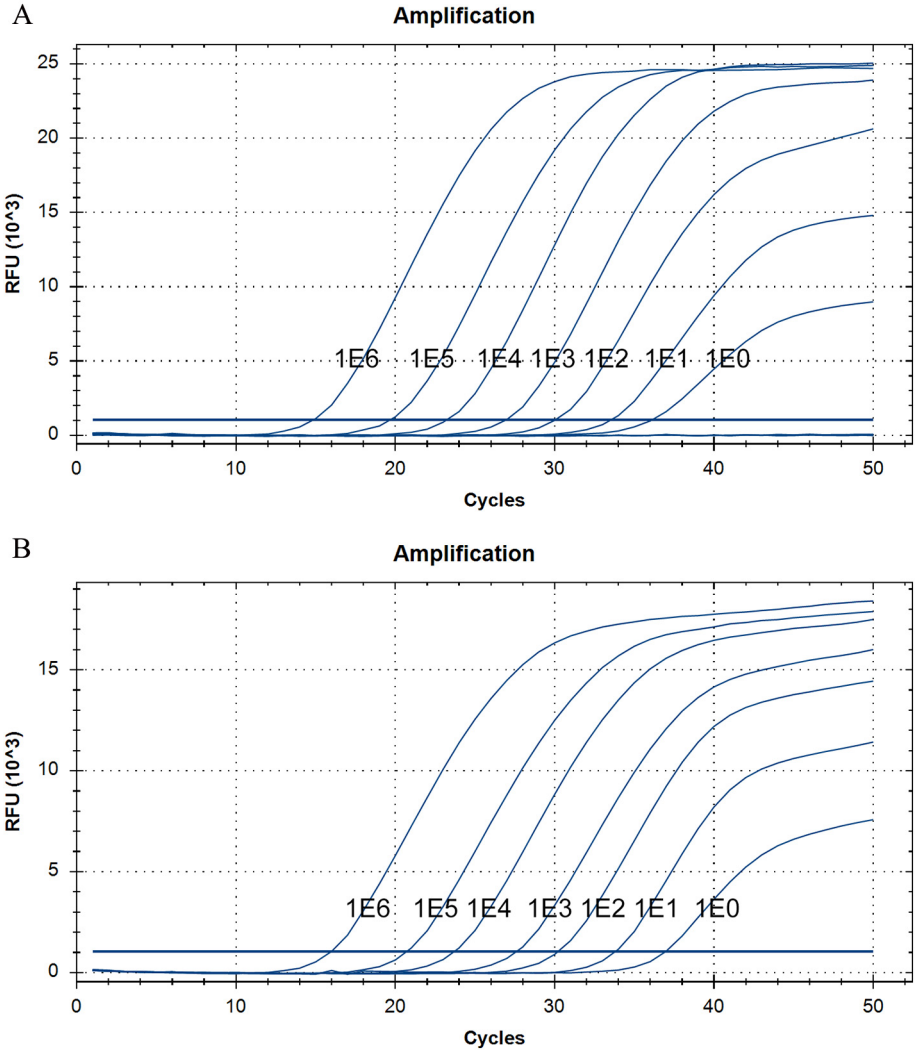

Fig. 2. Typical amplification plot derived from 10-fold dilutions of SARS-CoV-2 RNA transcripts. Typical amplification plot for the N gene assay (A) and the ORF 1b gene assay (B) against ten-fold dilutions of SARS-CoV-2 RNA transcripts. The plots were derived from dilutions containing 1×10^6 of one transcript copy for the N gene assay and the ORF 1b gene assay, respectively.

Table 3
Reproducibility of the TaqMan real-time RT-PCR assays.

Gene		Copy number of RNA						
		1×10^6	1×10^5	1×10^4	1×10^3	1×10^2	1×10^1	1×10^0
N	CV within assay (%)	0.52	1.33	0.33	0.46	0.20	1.25	0.78
	CV between assay (%)	1.06	2.45	1.49	1.32	1.37	1.45	0.77
ORF 1b	CV within assay (%)	0.73	0.26	1.10	1.30	4.45	3.36	–
	CV between assay (%)	4.66	3.85	2.77	2.17	5.12	3.50	–

Table 4
Results of the TaqMan real-time RT-PCR assays with specimens from suspected COVID-19 patients.

Patient ID	Specimen type	Ct value (copy number/ml)	
		N gene assay	ORF 1b gene assay
TS98	Throat swab	36.45 (6.50E+01)	Neg
TS101	Throat swab	33.96 (2.60E+02)	Neg
TS105	Throat swab	32.00 (4.14E+03)	Neg
TS108	Throat swab	34.07 (2.53E+02)	33.27 (3.70E+03)
TS110	Throat swab	30.24 (5.52E+04)	33.45 (3.56E+03)
TS165	Throat swab	27.26 (7.85E+05)	31.01 (5.50E+04)
TS169	Throat swab	33.82 (2.73E+02)	Neg
TS187	Throat swab	34.99 (1.80E+02)	Neg
TS188	Throat swab	35.42 (1.48E+02)	Neg
TS189	Throat swab	31.50 (4.53E+03)	34.27 (2.91E+03)
TS190	Throat swab	33.35 (3.08E+03)	Neg
TY1	Sputum	27.65 (7.55E+05)	31.27 (5.29E+04)
TY2	Sputum	29.79 (5.87E+04)	32.72 (4.14E+03)
TY3	Sputum	32.44 (3.80E+03)	36.23 (1.35E+02)
TY4	Sputum	23.22 (1.10E+06)	27.77 (8.07E+05)
TY6	Sputum	25.94 (8.39E+05)	29.39 (6.79E+04)
TY7	Sputum	27.19 (7.91E+05)	30.38 (6.00E+04)
TY8	Sputum	29.51 (6.09E+04)	34.05 (3.09E+03)
TY9	Sputum	27.00 (8.06E+05)	31.27 (5.29E+04)
XT1	Sputum	26.06 (8.79E+05)	30.17 (6.17E+04)
XT2	Sputum	32.29 (3.91E+04)	34.64 (2.63E+02)
XT3	Sputum	31.60 (4.45E+04)	35.34 (2.05E+02)
XT4	Sputum	33.11 (3.27E+04)	34.69 (2.58E+02)

gene assay, with lower efficiency in water (108%, slope = −3.08) than in the presence of RNA from other respiratory viruses (118%, slope = −2.92) (Table 2). These results suggested that the amplification reaction was more efficient in the presence of RNA from other respiratory viruses.

3.3. The specificity of the assays

To further evaluate the potential cross-reactivity with other human respiratory viruses, the developed TaqMan RT-PCR assays were examined using human respiratory samples positive for human coronaviruses (NL63, OC43, 229E, and HKU1), influenza A and B, respiratory syncytial virus, parainfluenza 1 to 4, human metapneumovirus, rhinovirus, adenovirus, and bocavirus. No cross reactivity was detected between the above viral nucleic acids for any of the developed RT-PCR assays (data not shown), demonstrating the high specificity of the developed assays.

3.4. Assay evaluation with clinical specimens

The developed real-time RT-PCR assays were applied to the testing of 23 clinical specimens (including 11 throat swabs and 12 sputum specimens) from 23 suspected COVID-19 patients (Table 4, Fig. S1). The N gene assay and the ORF 1b gene assay could detect SARS-CoV-2 from 100% (23/23) and 62.5% (16/23) of the clinical specimens, respectively. The ORF 1b gene assay could detect SARS-CoV-2 from 100% (12/12) of sputum samples and 36.4% (4/11) of throat swabs. The RNA copy number of the 23 samples were between 6.50E+01 and 1.10E+06 copies/ml calculated by the standard curve of ten-fold diluted N gene transcript, and from 2.05E+02 to 8.07E+05 copies/ml by that of ORF 1b gene transcript (Table 4). All of the seven specimens for which detection was missed by the ORF 1b gene assay were throat swabs, suggesting a lower vial load in the upper respiratory tract and the relatively lower sensitivity of the ORF 1b gene assay. The Ct value of the N gene assay was lower than that of the ORF 1b gene assay for each specimen, indicating the relative higher amplification efficiency of the N gene assay. These results suggested that the N gene assay performed better than the ORF 1b gene assay with higher sensitivity and amplification efficiency.

4. Discussion

In response to the outbreak of SARS-CoV-2-associated pneumonia, we developed two TaqMan real-time RT-PCR assays targeting the N gene and ORF 1b gene of the viral genome. Detection limits of one and ten transcript copies per reaction were achieved by the N gene assay and the ORF 1b gene assay, respectively. Low inter- and intra-assay variability and limited cross-reactivity with RNA from other respiratory viruses demonstrated the potential for quantitation over a wide dynamic range. The analytical performances suggested that the developed TaqMan real-time RT-PCR assays provide alternatives to aid the detection of SARS-CoV-2 at the stages of infection for which there is a lower viral load in respiratory secretions.

Rapid and reliable assays for SARS-CoV-2 detection are essential for the diagnosis of COVID-19 [9]. Due to the relatively lower viral load of SARS-CoV-2 in the upper respiratory tract, false negative results have often been observed during viral detection from throat swabs [10]. However, sampling from the lower respiratory tract is not feasible for the majority of patients with mild COVID-19 because of limited sputum production. Therefore, other methodologies, such as immunology methods (including antigen and antibody detection) and deep sequencing, were recommended to improve diagnostic accuracy of COVID-19 [6]. The simultaneous detection of SARS-CoV-2 by parallel viral detection assays targeting different genes may also be beneficial to the diagnostic accuracy of COVID-19 [5]. Thus, the development of more detection assays for SARS-CoV-2 has been recommended [11].

Although the TaqMan real-time RT-PCR assay is sensitive for virus detection [5,12], caution is needed when interpreting results with a Ct value higher than 35. False positive results could arise from slight mismatches between the primer/probe and template from the human genome or other sources. We addressed this by confirming the results by performing a repeat test when a weak positive result with a Ct value higher than 35 was obtained. The sensitivity of the N gene assay was 250 copies/ml, which were comparable to the commercial kits with sensitivities ranging from 1×10^2 to 1×10^3 copies/ml.

Because heat inactivation could affect the subsequent viral detection especially for those samples with low virus load, we did not perform heat activation but treated all the clinical specimens in a Biosafety Level 3 laboratory to ensure biosafety. Operating tests in high-throughput settings under severe pressure is error prone, thus the robustness of the operational procedures is crucial including positive and negative controls and the careful review of results. The quality of the sample is also crucial and it should be taken into consideration that the time point of sampling and the sampling technique could significantly affect the viral quantity in clinical samples.

In conclusion, we developed two TaqMan real-time RT-PCR assays permitting the rapid, sensitive and specific detection of SARS-CoV-2 in clinical specimens, thereby providing diagnostic support during the recent COVID-19 outbreak.

Ethics statement

This study was approved by the Ethics Review Committee of Jin Yin-tan Hospital (KY-2020-01.01) and the methods were performed in accordance with the approved guidelines.

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Conflict of interest statement

The authors declare that there are no conflicts of interest. Given their roles as Editorial Board Member, Jianwei Wang had no involvement in the peer-review of this article and had no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to the editor Guizhen Wu.

Author contributions

Yiwei Liu: Data curation, Formal analysis, Writing – original draft. **Yingying Wang:** Investigation. **Xinming Wang:** Formal analysis, Investigation. **Yan Xiao:** Formal analysis, Investigation. **Lan Chen:** Investigation. **Li Guo:** Investigation. **Jianguo Li:** Data curation, Formal analysis, Writing – original draft. **Lili Ren:** Conceptualization, Data curation, Formal analysis, Writing – review & editing, Funding acquisition. **Jianwei Wang:** Conceptualization, Writing – review & editing, Funding acquisition.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bsheal.2020.07.009.

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