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Short Communication

Clinical validation of an automated reverse transcription-insulated isothermal PCR assay for the detection of severe acute respiratory syndrome coronavirus 2

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Abstract To determine clinical performance of the single-target SARS-CoV-2 *orf 1 ab* reverse transcription-insulated isothermal PCR (RT-iiPCR) assay, the positive percentage agreement between this assay and a laboratory real-time RT-PCR assay was 96.8% (30 of 31; 95% confidence interval [CI], 90.5%–100%) and the negative percentage agreement was 97.1% (67 of 69; 95% CI, 93.1%–100%).

Introduction

Coronavirus disease 2019 (COVID-19) is an emerging viral respiratory disease that was first identified in Wuhan, China, in December 2019.[1] As of November 26, 2020, COVID-19 is an

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ongoing pandemic disease and affected more than 215 countries and territories, and more than 59 million cases and 1.4 million deaths.[2] The clinical manifestations of COVID-19 include asymptomatic, mild flu-like symptoms, loss of sensation of smell; pneumonia and acute respiratory distress syndrome are possible complications.[3,4] The duration of incubation period for COVID-19 is approximately 5 days,[5] and the important strategies to control the pandemic disease of COVID-19 include individual quarantine and active monitoring and testing of individuals. Currently, there are no antivirals of proven clinical efficacy for COVID-19.[6] Only remdesivir may shorten the time to recovery in adults hospitalized with lower respiratory tract infection.[7,8]

The etiological agent of COVID-19 is severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a member of the *Betacoronavirus* genus in Coronaviridae family. Following the emergence of SARS-CoV-2, several molecular detection methods and serological assays were quickly developed and deployed internationally through an international collaborative laboratory response.[9,10] Currently, real-time RT-PCR (RT-PCR) is the primary method for laboratory diagnosis of SARS-CoV-2 infection; however, RT-PCR assays are costly, demand expensive instrumentation, and require a dedicated laboratory environment with technically skilled personnel.[11] Consequently, simple and rapid methods are required to meet the needs of near-point-of-need SARS-CoV-2 detection. Fluorescent probe hydrolysis-based insulated isothermal PCR (iiPCR) for amplification and detection of nucleic acid has been described.[12] Based on these technologies, a compact and user-friendly iiPCR system, namely the POCKIT™ Nucleic Acid Analyzer series (GeneReach Biotech, Taichung, Taiwan) became available.[13,14] Various iiPCR assays for different pathogens in different hosts have been demonstrated previously to achieve sensitive and specific detection of target nucleic acid; specifically, performance of iiPCR for human pathogens including malaria, zika virus, Middle East respiratory syndrome coronavirus (MERS-CoV), dengue virus and malaria has been demonstrated to be equivalent to those of reference real-time PCR methods in different studies.[15—17] The bench-top POCKIT™ Central Nucleic Acid Analyzer automates magnetic bead-based nucleic acid extraction as well as fluorescence-based iiPCR amplification and detection to offer a simple sample-in-answer-out protocol. Qualitative results are available in less than 1.5 h. Taking advantage of this system, one single-target RT-iiPCR assay, namely POCKIT™ Central SARS-CoV-2 (*orf 1 ab*) Premix Reagent, targeting *orf 1 ab* for the detection of viral RNA was available commercially. Clinical performance of the assay was further evaluated and validated using oropharyngeal swab samples of patients with acute or chronic respiratory illness in recent outbreaks in Taiwan. The assay was compared to a routine laboratory real-time RT-PCR assay approved by the Taiwan Centers for Disease Control.

Material and methods

Clinical specimens

A total of 100 oropharyngeal swab samples were collected from patients with symptoms similar to those of COVID-19 at the Tauyuan General Hospital (TYGH) between March and April in 2020. The samples were collected in Sigma Virocult (Mini MW951S, Medical Wire Instrument, Corsham, UK). These included 45 male and 29 female subjects. Among them, 69 samples were collected from 58 patients suffering from known acute or chronic respiratory illnesses and previously tested negative for SARS-CoV-2 RNA. Additional 31 samples were collected from 16 patients with confirmed COVID-19 and previously being tested SARS-CoV-2 positive with a real-time RT-PCR assay (Table 1). The samples were tested first with the real-time RT-PCR assay. After the results were available, residual samples were subjected to the SARS-CoV-2 orf 1 ab RT-iiPCR assay.

Automated SARS-CoV-2 *orf 1 ab* RT-iiPCR assay

The RT-iiPCR assay [POCKIT™ Central SARS-CoV-2 (*orf 1 ab*) Premix Reagent, GeneReach Biotech] was an assay designed to detect *orf 1 ab* gene of SARS-CoV-2 RNA and an internal control simultaneously on the POCKIT™ Central Nucleic Acid Analyzer (GeneReach Biotech). The primers and probe for SARS-CoV-2 *orf 1 ab* were based on those released by the National Institute for Viral Disease Control and Prevention, China Center for Disease Control and Prevention.[15] The amplicon is located at the junction between *orf1a* and *orf1b* (nt 13331—13449, MT712863.1, isolate SARS-CoV-2/human/USA/CA-IGI-0045/2020), spanning 3′-end of *orf10, orf11 and* 5′-end of *orf12* (*RdRp* gene) regions. POCKIT™ Central Nucleic Acid Analyzer completes both nucleic acid extraction and RT-iiPCR. To run the assay, the Premix tube was placed into the designated well in the Transfer Cartridge. After adding 200 μL of the clinical sample into well one of the Extraction Cartridge, the operator logged information of the sample and reagent into the device, placed the cartridges onto the POCKIT™ Central device accordingly, and started the program. Fluorescence signals from fluorogenic probe hydrolysis chemistry before and at the end of the RT-iiPCR generated were collected through 520-nm and 550-nm channels for SARS-CoV-2 target and internal control, respectively. Data were processed and interpreted automatically by the POCKIT™ Central Nucleic Acid Analyzer to provide qualitative results in 85 min.

Reference SARS-CoV-2 real-time RT-PCR

The real-time RT-PCR assay developed by Corman et al., and listed in the World Health Organization (WHO) resource of in-house-developed molecular assays has been used as the national test in Taiwan and routinely performed at the TYGH's laboratory.[19,20] The assay included one or more reactions targeting the viral envelope (*E*), nucleocapsid (*N*), RNA-dependent RNA polymerase (*RdRp*) genes (Ref), as well as an internal control gene. The assay could be performed as a two-target system, where the E gene reaction detected various coronaviruses and the *RdRp* gene only detected SARS-CoV-2.[21] Briefly, 300 ul of oropharyngeal swab samples were first subjected to nucleic acid extraction by using the Labturbo kit (Taigen Bioscience, Taipei, Taiwan) on a LabTurbo 48 Compact System (Taigen Bioscience, Taipei, Taiwan) as described by the manufacturer. The isolated nucleic acids were eluted in 50 ul

Table 1 Detection of SARS-CoV-2 by RT-iiPCR and real-time RT-PCR in oropharyngeal swab samples collected from patients infected with COVID-19.

Patient's ID	Age	Gender	Date of onset	Outcome	Collection date	SARS-CoV-2 RT-iiPCR	Real-time PCR (Ct value)	
						orf 1 ab	E gene	RdRp gene
TYGH-1	59	M	09 Apr. 2020	Recovered	16 Apr. 2020	+	21.7	26.2
					17 Apr. 2020	−	33.6	36.9
TYGH-2	67	M	05 Apr. 2020	Recovered	20 Apr. 2020	+	35.1	40.0
TYGH-3	42	M	09 Apr. 2020	recovered	17 Apr. 2020	+	24.2	27.9
TYGH-4	29	F	05 Apr. 2020	recovered	16 Apr. 2020	+	34.3	40.0
					17 Apr. 2020	+	31.7	37.3
TYGH-5	29	M	13 Apr. 2020	recovered	22 Apr. 2020	+	30.6	33.4
					28 Apr. 2020	+	29.8	31.9
TYGH-6	22	F	15 Apr. 2020	recovered	20 Apr. 2020	+	18.9	23.6
					27 Apr. 2020	+	32.3	37.3
TYGH-7	26	M	15 Apr. 2020	recovered	21 Apr. 2020	+	18.5	21.6
					22 Apr. 2020	+	25.2	27.9
					28 Apr. 2020	+	31.0	32.8
TYGH-8	22	F	19 Apr. 2020	recovered	20 Apr. 2020	+	14.5	17.8
					22 Apr. 2020	+	16.5	18.2
					28 Apr. 2020	+	27.5	31.0
TYGH-9	25	M	17 Apr. 2020	recovered	20 Apr. 2020	+	31.0	36.6
					22 Apr. 2020	+	29.7	33.6
					28 Apr. 2020	+	30.2	33.1
TYGH-10	41	M	18 Apr. 2020	recovered	20 Apr. 2020	+	30.9	35.8
					22 Apr. 2020	+	30.9	34.4
TYGH-11	37	M	14 Apr. 2020	recovered	20 Apr. 2020	+	21.2	26.5
					22 Apr. 2020	+	30.9	35.4
					28 Apr. 2020	+	33.0	35.1
TYGH-12	31	M	15 Apr. 2020	recovered	21 Apr. 2020	+	20.7	23.7
TYGH-13	36	F	05 Apr. 2020	recovered	28 Apr. 2020	+	32.0	36.5
TYGH-14	21	F	20 Mar. 2020	recovered	16 Apr. 2020	+	30.9	36.4
					17 Apr. 2020	+	28.9	31.4
TYGH-15	63	F	21 Mar. 2020	recovered	16 Apr. 2020	+	31.8	38.3
					17 Apr. 2020	+	34.9	38.4
TYGH-16	65	F	24 Mar. 2020	recovered	17 Apr. 2020	+	33.5	40.0

Elution Buffer. Real-time RT-PCR was performed with the LightCycler Multiplex RNA Virus Master Kit (Roche Diagnostics, Mannheim, Germany) on the cobas z480 analyzer (Roche Diagnostics, Mannheim, Germany). Primers and probes were purchased from ModularDx Kit (Tib-Molbiol, Berlin, Germany). Negative samples had Ct values of higher than 37 in both *E* gene and *RdRp* gene reactions, but E gene CT value of <37 and RdRp gene CT value of between 37 and 40 was defined very low viral load.

Statistical analysis

The degree of agreement between the two assays was assessed by kappa analysis using SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA). Positive percent agreement was calculated as (number positive by both methods)/(number positive by reference method) and negative percent agreement was calculated as (number negative by both methods)/(number negative by reference method).

Ethics statement

Clinical data and specimens were obtained from the patients with ethical approval granted by the Institutional Review Board of Taoyuaral Hospital, Taoyuan City, Taiwan (TYGH109016). All patients provided written informed consent before participating in the study. All experiments were performed according to the approved protocol.

Results

To determine clinical performance of the single-target SARS-CoV-2 *orf 1 ab* RT-iiPCR assay, 31 oropharyngeal swab samples from 16 patients during the course of COVID-19 illness (group A) and 69 samples from 58 patients suffering from other known respiratory illnesses (group B) were analyzed during COVID-19 outbreaks in Taiwan in 2020. The samples were subjected to side-by-side testing with the SARS-CoV-2 *orf 1 ab* RT-iiPCR assay and the national

laboratory real-time RT-PCR assay. The samples were tested first with the real-time RT-PCR assay. After the results were available, residual samples were subjected to the SARS-CoV-2 *orf 1 ab* RT-iiPCR assay. In total, 30 of 31 (group A) and 2 of 69 (group B) samples were positive and 1 of 31 (group A) and 67 of 69 (group B) were negative by the RT-iiPCR assay, and 31 samples were positive and 69 samples were negative by the reference real-time RT-PCR assay.

2 × 2 contingency analysis showed that 30 samples were positive and 67 were negative with both assays. One sample tested positive by the reference assay but negative by the RT-iiPCR assay; two samples tested negative by the reference assay but positive by the RT-iiPCR assay. The positive percentage agreement between the two assays was 96.8% (30 of 31; 95% confidence interval [CI], 90.5%—100%) and the negative percentage agreement was 97.1% (67 of 69; 95% CI, 93.1%—100%). Lastly, the overall percentage agreement between the two assays was 97.0% (95% CI, 93.6%—100%) with a kappa value of 0.93, indicating that the two methods had comparable clinical performance.

Discussion

In this study, the SARS-CoV-2 *orf 1 ab* RT-iiPCR assay was shown to have performance comparable to that of the reference laboratory real-time RT-PCR assay by side-by-side testing of oropharyngeal swab samples collected from patients with acute or chronic respiratory illness including patients who previously tested positive for SARS-CoV-2. High positive agreement (96.8%) and high negative agreement (97.1%) were found between the two assays. Discrepancy between two different assays are most likely due to that the target was present at low titers. However, further analysis of the discrepant samples was not possible because the amounts of the samples remained were too low. Testing of more clinical samples is being coordinated to further support the clinical specificity and sensitivity of the *orf 1 ab* RT-iiPCR.

The POCKIT™ Central iiPCR system qualitative results are available for 8 samples in less than 1.5 h after the run start. Real-time RT-PCR, being able to process at least 96 tests in one run, has in general higher throughput than the POCKIT™ Central system. However, with the needs for an additional step for nucleic acid extraction which took about 1.5 h, and for testing at least two SARS-CoV-2 genes to aid COVID-19 diagnosis, the laboratory real-time RT-PCR testing protocol took about 3—4 h to provide results for 30 samples.

The reference real-time RT-PCR assay was designed to test at least 2 viral genes to ensure reactivity for SARS-COV-2 since only few sequences of the virus became available at the time of assay development.[11] Recently, based on increasing numbers of SARS-CoV-2 sequences available, it is generally reckoned that single viral target SARS-CoV-2 assay with proper validation could meet the needs of laboratory diagnosis.[22] The SARS-CoV-2 RT-iiPCR target only the *orf 1 ab* gene described in the China CDC real-time RT-PCR assay.[18] The sequences are located between the *NSP11* and *NSP12* (*RdRP*) genes. No mutations were found in 7818 SARS-CoV-2 genome sequences as of May 1, 2020 in the primers and probe sequences (ORF1ab-China-F, ORF1ab-China-R, and ORF1ab-China-P) of the China CDC method.[23] In addition, various potential mutation hotspots identified in SARS-CoV-2 genome are not located in the close proximity of the *orf 1 ab* primer and probe sequences, according to analysis of large-scale SARS-CoV genomic sequences.[24,25]

The data presented in this study demonstrated that the performance of the single-target SARS-CoV-2 *orf 1 ab* RT-iiPCR assay was comparable to that of the reference real-time RT-PCR assay while was able to provide much faster results than the real-time RT-PCR.

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Declaration of competing interest

All authors report no conflicts of interest.

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