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ORIGINAL RESEARCH

Comparative evaluation of a dual-target real-time RT-PCR assay for COVID-19 diagnosis and assessment of performance in pooled saliva and nasopharyngeal swab samples

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ABSTRACT

Objectives: Sensitive molecular diagnostic assays are essential for COVID-19 diagnosis. We evaluated the Hecin Scientific SARS-CoV-2 nucleic acid test kit, a dual-target real-time RT-PCR assay targeting the SARS-CoV-2 N and ORF1ab genes.

Methods: The Hecin test kit's diagnostic performance in detecting SARS-CoV-2 RNA was compared to the LightMix Modular SARS and Wuhan CoV E-gene kit (TIB Molbiol) and an in-house single-tube nested real-time RT-PCR using 296 clinical specimens, 11 proficiency testing samples, and 30 low-positive deep throat saliva and nasopharyngeal swab (NPS) samples pooled into negative samples in ratios of 1:5, 1:10, and 1:30.

Results: The limit-of-detection of the Hecin test kit was around 500 dC/mL for the N and ORF1ab targets. Sensitivity and specificity of the Hecin test kit were 98.1% (95% CI: 93.4–99.8%) and 100% (98.1–100%), respectively, when measured against the reference method. The Hecin test kit showed fair sensitivity (80%) in low-positive NPS samples pooled in ratios of 1:5 and 1:10. Its performance in pooled samples could be dramatically improved by adjusting the assay Ct cutoff.

Conclusion: The Hecin test kit enables sensitive and specific detection of SARS-CoV-2 in clinical samples and pooled samples.

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SARS-CoV-2; COVID-19; real-time RT-PCR; diagnostic test evaluation; pooling

1. Introduction

Coronavirus disease 2019 (COVID-19) is caused by SARS-CoV-2, a newly emerged betacoronavirus [1,2]. COVID-19 has affected human life and activity more than any other pandemic in modern history. As of 27 April 2021, there have been 146 million confirmed COVID-19 cases, of which 3 million people have died (https://www.who.int/emergencies/diseases/novel-coronavirus-2019), although actual numbers of affected individuals are likely much higher. SARS-CoV-2 RNA detection by real-time reverse-transcription polymerase chain reaction (RT-PCR) is the most commonly used diagnostic tool for COVID-19 [3–6]. Sensitive SARS-CoV-2 RT-PCR assays are not only important for patient diagnosis, but also facilitate community-level outbreak control and containment [7]. There is increased demand globally for RT-PCR reagents, which has raised the threat of shortage, especially for low- and middle-income countries [8,9]. One approach to tackle this problem is to pool samples together for extraction followed by RT-PCR testing [10]. Pool positivity would then trigger testing of individual samples. Concerns of reduced sensitivity with pooling have been raised and mathematical algorithms for calculating optimal pool size have been proposed [11–13]. Furthermore, the best sample types and optimal RT-PCR assay design for pooled testing are unknown. This study had two objectives: we first evaluated the performance of a novel dual-target commercial kit for COVID-19 diagnosis and compared its performance to the LightMix E-gene kit and our previously validated in-house single-tube nested (STN) COVID-19-N RT-PCR assay [14,15]. We then examined the sensitivity of pooled testing of paired nasopharyngeal swab (NPS) and deep throat saliva samples in a range of pool sizes using three RT-PCR assays.

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Supplemental data for this article can be accessed here

2. Patients and methods

2.1. Viruses, clinical specimens, and proficiency samples for evaluation

Serial twofold dilutions of total nucleic acid (TNA) extracted from a Qnostics SARS-CoV-2 Medium Q Control (RANDOX, UK) were used for analytical sensitivity or limit-of-detection (LOD) evaluation. Different concentrations of TNA extracted from a SARS-CoV-2 culture isolate stock (1.8×10^7 $TCID_{50}$/mL) isolated from the nasopharyngeal aspirate (NPA) of a patient with COVID-19 in Hong Kong were used for imprecision evaluation [16–18]. Each concentration was tested in triplicate in single run for intra-assay, and tested over two independent runs (each run with triplicate) for inter-assay imprecision evaluation. TNAs extracted from a clinical respiratory specimen positive for human coronavirus HKU1 (HCoV-HKU1) and 17 culture isolates of SARS-CoV, MERS-CoV, HCoV-OC43, HCoV-229E, HCoV-NL63, influenza A ((H1N1)pdm09 and H3N2) viruses, influenza B virus, influenza C virus, respiratory syncytial virus, human metapneumovirus, parainfluenza virus types 1–4, human rhinovirus, and human adenovirus were used for analytical specificity evaluation [19]. For diagnostic performance evaluation, we retrieved 296 clinical specimens obtained for initial diagnostic evaluation of hospitalized patients with suspected COVID-19. These included 255 respiratory tract specimens (NPA, NPS, throat swab, deep throat saliva or sputum) and 41 non-respiratory specimens (conjunctival swab, rectal swab, or stool). The male: female ratio was 136:160 and patients' median age was 59.5 years (range: 3 months – 99 years). Some of these specimens were previously used in other assay evaluations published by our group [14,15,20]. In addition to clinical specimens, three proficiency testing (PT) samples from College of American Pathologist (CAP) and eight PT samples from Quality Control for Molecular Diagnostics (QCMD) with different concentrations of SARS-CoV-2 or negative for SARS-CoV-2 were also evaluated.

To evaluate the practicality of pooling clinical specimens for COVID-19 diagnosis and deep throat saliva as an alternate specimen type for pooling, we selected 15 paired NPS and saliva specimens obtained on the same day from 15 hospitalized patients with confirmed COVID-19. Each positive specimen had a low viral load (defined as Ct > 30 by the LightMix E-gene kit, which is the test used in routine clinical practice in our laboratory) and was pooled with SARS-CoV-2-negative specimens in three different ratios (5 pooled in 1, 10 pooled in 1, and 30 pooled in 1). For example, pool of 5 indicates one positive sample spiked into four negative samples. For all three pooling ratios, 50 µL of each sample were added in each pool, and 250 µL of each well-mixed pool was subjected to TNA extraction. These pooled samples were evaluated by three different real-time RT-PCR assays as described below. The present study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (UW 20–286).

2.2. Nucleic acid extraction

We performed TNA extraction on clinical specimens, pooled samples, PT samples, and virus culture isolates using the NucliSENS easyMAG extraction system (bioMérieux, Marcy-l'Étoile, France) as previously described [21]. Briefly, 250 µL of each sample was added into a lysis buffer, and 55 µL of eluate was obtained and stored at −80°C until use.

2.3. Real-time RT-PCR assays for SARS-CoV-2 RNA detection

The performance of a commercial dual-target SARS-CoV-2 nucleic acid test kit (Hecin Scientific, Inc., Guangdong, China) was evaluated in the present study. The Hecin test kit is a dual-target real-time RT-PCR assay that can detect the N and ORF1ab regions of SARS-CoV-2 and a human gene target as an internal control [22]. Each 20 µL reaction mixture contained 5 µL of SARS-CoV-2 amplification reaction solution 1 (primers, probes, PCR reaction buffer, and dNTPs), 5 µL of SARS-CoV-2 amplification reaction solution 2 (enzyme mixture), and 10 µL of TNA as the template. Amplification and detection were performed using LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland). FAM, VIC and Texas Red channels were chosen for detection of SARS-CoV-2 N-gene target, SARS-CoV-2 ORF1ab target, and human gene (as internal control), respectively. The thermocycling condition was 48°C for 5 min, 94°C for 2 min, and 45 cycles of 94°C for 10 s and 60°C for 35 s. The test kit consisted of a SARS-CoV-2 positive control (pseudoviral particles including ORF1ab and N gene target sequences) and a negative control (inactivated human cell culture) that were subjected to TNA extraction for monitoring the assay performance. For the resulting interpretation, samples are regarded as positive when Ct ≤ 37 for both N and ORF1ab, while samples are regarded as highly suspected positive when one gene target with Ct ≤ 37 and another gene target with Ct > 37 or undetermined. Repeated testing is recommended for these samples. If the results in the repeated testing showed that both gene targets were positive or either target was positive (Ct ≤ 37), then the samples are confirmed to be true positive. Samples are regarded as negative when Ct > 37 or undetermined for both gene targets and Ct ≤ 37 for internal control according to manufacturer's instructions.

Two validated comparator real-time RT-PCR assays for SARS-CoV-2 detection were used in this study. LightMix Modular SARS and Wuhan CoV E-gene kit (TIB Molbiol, Berlin, Germany) with LightCycler Multiplex RNA Virus Master (Roche) was used as we previously described [14] and was designated as the reference method. Briefly, each 20 µL reaction mixture contained 4 µL of Roche Master, 0.5 µL of reagent mix, 0.1 µL of RT Enzyme, 5.4 µL of water, and 10 µL of TNA as the template. RT-PCR was performed using the LightCycler 480 II Real-Time PCR System (Roche). The thermocycling condition consisted of 55°C for 5 min, 95°C for 5 min, followed by 45 cycles of 95°C for 5 s, 60°C for 15 s, and 72°C for 15 s.

The second assay is an in-house developed STN real-time RT-PCR assay targeting the SARS-CoV-2 N gene was performed using QuantiNova Probe RT-PCR Kit (QIAGEN, Hilden, Germany) on LightCycler 480 II Real-Time PCR System (Roche) previously described by us [15]. This assay format maximizes sensitivity by combining two PCR reactions within a single reaction vessel. Each 20 µL reaction mixture contained 10 µL of 2× QuantiNova Probe RT-PCR Master Mix, 0.2 µL of QN Probe RT-Mix, 0.16 µL of each 10 µM outer forward and reverse primer, 1.6 µL of each 10 µM inner forward and reverse primer, 0.4 µL of 10 µM probe, 0.88 µL of nuclease-free water and 5 µL of TNA as the template. The thermocycling condition was 45°C for 10 min, 95°C for 5 min, followed by 20 cycles of 95°C for 5 s and 69°C for 30 s, and then 40 cycles of 95°C for 5 s and 55°C for 30 s. The comparison of the characteristics of the three assays is summarized in Table S1.

2.4. Statistical analysis

Probit regression analysis was used to determine the detection limit of the Hecin test kit. Fisher's exact test was used to compare the performance of the assays or the detection rates between NPS and saliva for each pooling size. Spearman's correlation was used to assess the relation between the Ct values of different real-time RT-PCR assays. Ct values obtained from different assays or different pooling sizes of each specimen type were compared using ANOVA Friedman test with Dunn's multiple comparison tests (a Ct value of 41 was assigned to specimens that tested negative in the real-time RT-PCR assays). Ct values obtained from paired NPS and saliva pooled samples were compared using t tests (Wilcoxon matched-pairs signed rank test). $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad PRISM 8 or SPSS 26.0.

3. Results

3.1. Analytical performance of the Hecin test kit for SARS-CoV-2 detection

The LOD of the Hecin test kit was 528 dC/mL (95% CI: 313–1855 dC/mL) for N gene target and 532 dC/mL (95% CI: 347–1565 dC/mL) for ORF1ab target of SARS-CoV-2 (Table S2). For analytical specificity, the N and ORF1ab assays of the Hecin test kit did not cross-react with the above-mentioned respiratory viruses including human coronaviruses, indicating that the assays were specific to SARS-CoV-2. The intra- and inter-assay variations of the samples by the Hecin test kit were evaluated using different concentrations of TNA extracted from the SARS-CoV-2 isolate, the total imprecision (% CV) values ranged from 0.31% to 1.00% (Table S3).

Table 1. Comparison between the Hecin test kit and other validated real-time RT-PCR assays for SARS-CoV-2 detection in different specimen types from patients with suspected COVID-19.

Specimen type* (No. of specimens)	Number of positive results (%) Hecin test kit	LightMix E-gene kit	In-house STN COVID-19-N assay	*P*-value†
Respiratory tract:				
NPA/NPS/TS (128)	60 (46.9)	61 (47.7)	60 (46.9)	NS
Saliva (122)	35 (28.7)	36 (29.5)	36 (29.5)	NS
Sputum (5)	0 (0)	0 (0)	0 (0)	NS
Non-respiratory tract:				
Conjunctival swab (23)	3 (13.0)	3 (13.0)	3 (13.0)	NS
Rectal swab/ stool (18)	7 (38.9)	7 (38.9)	7 (38.9)	NS
Total (296)	105 (35.5)	107 (36.1)	106 (35.8)	NS

*Abbreviations: NPA, nasopharyngeal aspirate; NPS, nasopharyngeal swab; TS, throat swab
†NS, not significant

3.2. Diagnostic performance of the Hecin test kit for SARS-CoV-2 detection

To assess the diagnostic performance of the Hecin test kit in clinical specimens, 296 initial clinical specimens from patients with suspected COVID-19 were subjected to SARS-CoV-2 detection by the Hecin test kit and two other RT-PCR assays: the LightMix E-gene kit and our in-house developed STN COVID-19-N RT-PCR assay. Among the 296 specimens, 105 (35.5%) were positive for SARS-CoV-2 by the Hecin test kit, while 107 (36.1%) and 106 (35.8%) were positive for SARS-CoV-2 by the LightMix E-gene kit and the in-house STN COVID-19-N RT-PCR assay, respectively (Table 1). There was no significant difference in the detection rate between the Hecin test kit and the comparator assays ($P > 0.05$). Using the LightMix E-gene assay as the reference method, the diagnostic sensitivity and specificity of the Hecin test kit were 98.1% (95% CI: 93.4–-99.8%) and 100% (98.1–100%), respectively, while those of the in-house STN COVID-19-N assay were 99.1% (95% CI: 94.9–-99.9%) and 100% (98.1–100%), respectively (Table 2). Two clinical specimens that showed discordant results had low viral load: 1) one saliva tested positive by the LightMix E-gene kit (Ct: 36.84) and the STN COVID-19-N assay (Ct: 29.69) was negative by the Hecin test kit and 2) one NPS tested positive by the LightMix E-gene kit (Ct: 34.87) was negative by the Hecin test kit and the STN COVID-19-N assay

Table 2. Clinical performance comparison of the Hecin test kit and the in-house STN COVID-19-N assay with the LightMix E-gene assay as the reference method.

Molecular assays	LightMix E-gene assay		Sensitivity (95% CI)	Specificity (95% CI)
	Positive	Negative		
Hecin test kit				
Positive	105	0	98.1%	100%
Negative	2	189	(93.4–99.8%)	(98.1–100%)
STN COVID-19-N assay				
Positive	106	0	99.1%	100%
Negative	1	189	(94.9–99.9%)	(98.1–100%)

(Table S4). Among 105 specimens tested SARS-CoV-2-positive by the Hecin test kit, 7 (6.7%) showed sole positivity for the N-gene target (n = 5, Ct: 33.64–35.81) or ORF1ab-gene target (n = 2, Ct: 28.64–34.55) upon repeat testing, thereby fulfilling criteria for positivity according to manufacturer's instructions (Table S4). Among the 296 specimens, all showed positive for internal control with Ct ranging from 16.73 to 34.57, except one SARS-CoV-2-positive rectal swab showed negative. Among the three PT samples from CAP and eight PT samples from QCMD, the Hecin test kit gave 100% correct results. Agreements between Ct values generated by the Hecin test kit (N and ORF1ab targets) and the LightMix E-gene kit were demonstrated by strong correlation (Spearman's $\rho \geq 0.986$; $P < 0.0001$) (Figure 1). The Ct values obtained from the Hecin test kit and the LightMix E-gene kit were also examined. The median Ct values (interquartile range, IQR) of the Hecin test kit N target (29.66, IQR 24.17–33.02) and ORF1ab target (30.08, IQR 24.98–33.38) were significantly higher than that of the LightMix E-gene assay (28.30, IQR 23.48–32.60) ($P < 0.0001$) (Figure 2). The Ct values of the STN COVID-19-N RT-PCR assay were not compared to the Hecin test kit due to the differences in assay format (nested vs non-nested, which would not enable a fair comparison).

3.3. Evaluation of pooled NPS and deep throat saliva samples using three COVID-19 RT-PCR assays

To evaluate pooling strategies for COVID-19 diagnosis, each positive sample with low viral load (Ct > 30) were spiked into negative samples at pooling ratios of 1:5, 1:10 and 1:30. For each pool size, 15 pools of NPS and 15 pools of saliva were tested by three COVID-19 RT-PCR assays (Table S5). When results were interpreted according to manufacturer instructions, the Hecin test kit had equivalent detection rates to the comparator assays for both NPS and saliva samples, except for the pool of 10 saliva samples (Table 3). The detection rates could achieve 80% in pools of 5 and 10 for NPS tested positive by Hecin test kit. In most pooling conditions and detection formats, NPS pools performed better than saliva (Table 3). We reasoned that the stringent manufacturer cutoff for the Hecin test kit (requiring a Ct < 37) was a disadvantage when testing pooled samples. Therefore, we examined the effect of adjusting the Ct cutoff of the Hecin test kit to 39. Indeed, upon adjustment of the Hecin test kit positivity cutoff Ct to 39 in either one target, the detection rate improved to >90% for both NPS and saliva samples pooled 1:5 (Table 3).

To evaluate the reduction in viral load after pooling a positive sample with negative samples in different ratios, Ct values of pooled NPS and saliva samples were compared with those of the individual samples tested by the LightMix E-gene kit. For NPS samples, the median Ct (IQR) of the individual samples by LightMix E-gene kit was 33.28 (30.96–-35.65), which was significantly lower than those of the pools of 5 [35.6 (33.7–37.4)] (P = 0.0069), pools of 10 [36.73 (35.14–-37.41)] (P = 0.0024) and pools of 30 [41 (34.98–41)] (P < 0.0001). For saliva samples, the median Ct (IQR) of individual samples by LightMix E-gene kit was 34.51 (32.86–35.76), which was significantly lower than those of the pools of 5 [36

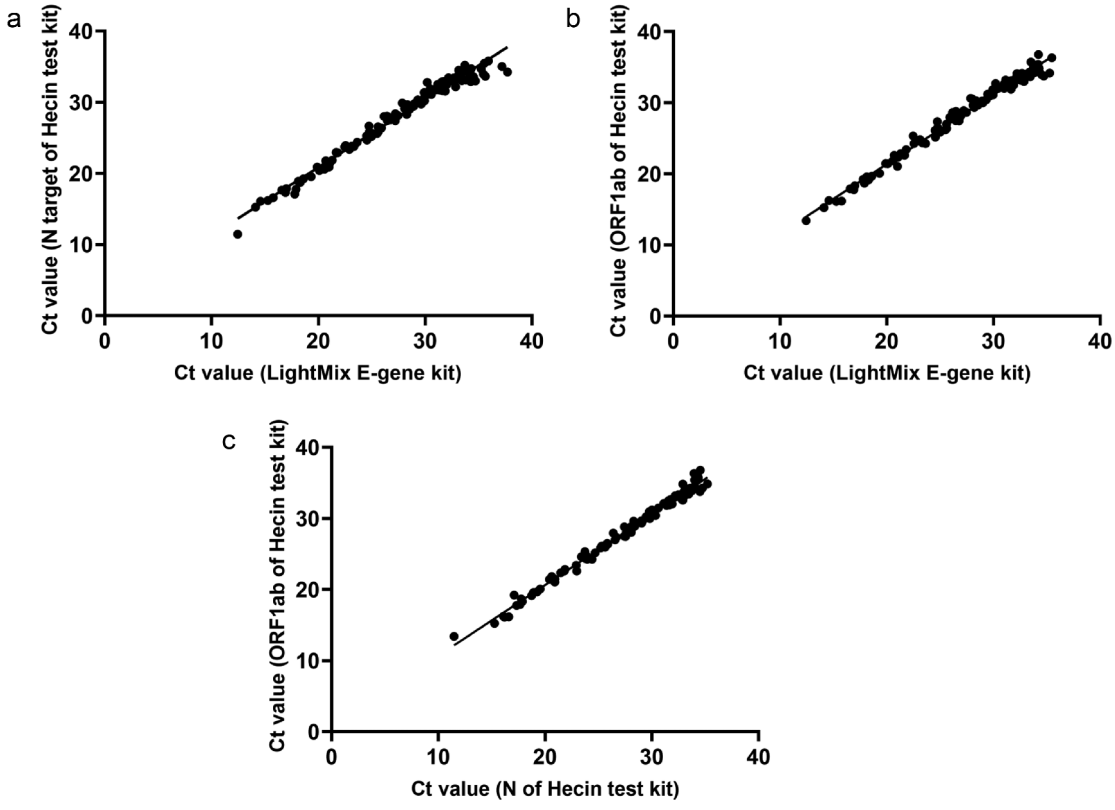

Figure 1. Correlation of the Ct values of the samples found positive for SARS-CoV-2 by the Hecin test kit and LightMix E-gene kit: (A) N target of Hecin kit vs E-gene kit, (B) ORF1ab target of Hecin kit vs E-gene kit and (C) N target vs ORF1ab target of Hecin kit.

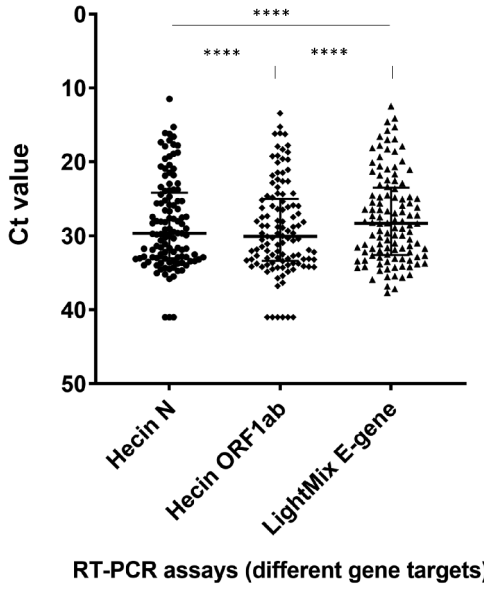

Figure 2. Comparison of the Ct values of the Hecin test kit and LightMix E-gene kit for samples positive according to the reference standard. A Ct value of 41 was assigned to samples that tested negative by either kit. **** indicates $P < 0.0001$.

(35.2–41)] (P = 0.0032), pools of 10 [36.11 (35.5–41)] (P = 0.0019) and pools of 30 [35.59 (35.49–41)] (P = 0.0041).

Ct values of the three pool sizes for each specimen type were compared across all three diagnostic formats. For both NPS and saliva specimen types, no significant difference in the median Ct values was noted between the pool of 5 and the pool of 10 by all three assays. For NPS, significant differences were noted between the pools of 5 and the pools of 30 by two assays [36.28 (35.13–37.65) vs 38 (37.7–41) for Hecin N (P = 0.003); 36.4 (34.97–41) vs 41 (37.17–41) for Hecin ORF1ab (P = 0.041); and 31.8 (28.9–35) vs 41 (31.99–41) for STN COVID-19-N (P < 0.001)], and between the pool of 10 and the pool of 30 [36.56 (35.1–38.55) vs 38 (37.7–41) for Hecin N (P = 0.008)]. For saliva, significant difference was noted between the pools of 5 [32.1 (27.6–35)] and the pools of 30 [41 (32.02–41)] by STN COVID-19-N assay (P = 0.032).

To determine if there was a significant difference in viral load between NPS and saliva specimen types, Ct values of pooled NPS and saliva samples in different pooling sizes by the three RT-PCR assays were compared. In each pool size category, no significant difference in the median Ct values was noted between NPS and saliva, except for the pools of 10, in which significant differences were noted between NPS and saliva by the Hecin test kit [36.56 (35.1–38.55) vs 37.74 (36.17–41) for N target (P = 0.049); 36 (35.34–41) vs 41 (36.-88–41) for ORF1ab target (P = 0.020)].

4. Discussion

In this study, we evaluated the Hecin test kit, a dual-target assay, for the detection of SARS-CoV-2 RNA. The incorporation of two SARS-CoV-2 specific test targets ensured excellent sensitivity and specificity. Detection of two targets also potentially eliminates the need of time-consuming confirmatory assays if both target reactions are flagged positive. The Hecin test kit also detects a human housekeeping gene in clinical specimens as an internal control, which allows monitoring of the specimen quality, the presence of inhibitors, efficiency of extraction, and amplification. The LightMix E-gene kit, on the other hand, uses an internal control derived from Equine Arteritis Virus, which needs to be added to each sample. This is inconvenient and may increase the risk of contamination. The reagent cost of the Hecin test kit is lower than that of the LightMix E-gene kit.

In our previous studies, we have evaluated various commercial and in-house developed RT-PCR assays for COVID-19 diagnosis [5,6,14,15,19,23]. Among these assays, the LightMix E-gene kit and in-house developed STN COVID-19-N RT-PCR assay were most sensitive. Therefore, we chose these two as comparator assays in this study. The Hecin test kit was highly sensitive for SARS-CoV-2 RNA detection, with the LOD of around 500 dC/mL for both N and ORF1ab gene targets. The median Ct values of the Hecin test kit (N and ORF1ab targets) in clinical specimens were significantly higher than the LightMix E-gene kit, this was probably because the Hecin test and LightMix E-gene assays were performed using different PCR reagents, thermocycling conditions and PCR machines that use different algorithms for Ct calculation. The differences in Ct values that were shown to be statistically significantly different were very small and were not biologically different. Nevertheless, no significant difference in the diagnostic performance was noted among these assays for both respiratory and non-respiratory specimens, as well as PT samples from multiple sources. One NPS (Ct: 34.87) and one saliva (Ct: 36.84) tested positive by the LightMix E-gene assay were negative by the Hecin test kit, this was probably due to low viral load in these two specimens. Altogether, these findings suggested that the Hecin

Table 3. Detection rates of the pooled NPS and deep throat saliva samples tested by three different COVID-19 RT-PCR assays.

Pooling size*	Specimen type (No. of pooled samples tested)	Number of positives (%) Hecin test kit (according to manufacturer's instructions)	Hecin test kit (with a cutoff Ct = 39 in either target)	LightMix E-gene kit	STN COVID-19-N assay
Pool of 5	NPS (15)	11 (73.3)	14 (93.3)	12 (80)	13 (86.7)
	Saliva (15)	10 (66.7)	15 (100)	11 (73.3)	12 (80)
Pool of 10	NPS (15)	12 (80)	14 (93.3)	14 (93.3)	12 (80)
	Saliva (15)	6 (40)	9 (60)	11 (73.3)	10 (66.7)
Pool of 30	NPS (15)	3 (20)	11 (73.3)	7 (46.7)	7 (46.7)
	Saliva (15)	6 (40)	9 (60)	9 (60)	6 (40)

*Each pool contained one positive sample spiked into samples negative for SARS-CoV-2 (e.g. pool of 5 indicates one positive sample spiked into 4 negative samples).

test kit showed good diagnostic performance for COVID-19 diagnosis.

Among 105 specimens positive for SARS-CoV-2 by the Hecin test kit, most of them were positive for both N and ORF1ab targets, while 7 were positive for either gene target. We found that one specimen with moderate viral load (Ct: 28.64) was solely positive for the ORF1ab target but negative for N target by the Hecin test kit, similar result was obtained after repeated testing. This raises the possibility of mismatches between the primer/probe sequences of the Hecin kit and the N gene sequences of the SARS-CoV-2 isolates in this specimen. Further investigation is warranted to determine if mutations have occurred in the N gene of this SARS-CoV-2 isolate.

Pooling of clinical specimens is a cost-effective method for screening of COVID-19 [10]. Pooling is particularly useful in mass screening programs [24–26], which allows early identification of infected asymptomatic persons [27]. However, pooling would certainly result in a lower detection rate for samples with high Ct value compared to individual sample testing. This is a disadvantage in settings where high-sensitivity testing is desirable such as hospitals. In this study, we evaluated pools of two sample types: NPS and deep throat saliva, pooled in three different ratios using three different assays. Our main findings are that a) detection rate of NPS pools generally tended to be better than saliva pools, b) the dual-target Hecin assay was equivalent to single-target assays including those utilizing nested formats in small pool sizes and c) the detection rate of the Hecin test kit for pooled samples could be improved by adopting a less stringent Ct cutoff, although this would result in some loss of specificity. With regard to this last finding, we found that the Hecin test kit cutoff criteria may result in a reduction in detection rate for pooled samples. Therefore, we propose using a modified cutoff Ct of 39 to maximize detection rates. As individual samples in positive pools would be retested by the same kit or another platform to identify the positive sample, the impact of false-positives arising from this adjusted cutoff can be minimized. As expected, we found that detection rates dropped with larger pool sizes. However, optimal pool size in real-life settings depends on the prevalence of SARS-CoV-2 infection and pooling of specimens is particularly useful and cost-effective when the positive rate is low (e.g. < 1%). Several studies demonstrated that a pool size of 5 or 10 is optimal when expected COVID-19 prevalence rate is around 5%, while a pool size of around 30 is optimal for the prevalence rate of 0.1% [10–13]. Lohse et al. demonstrated that Ct values of pools were lower than those of single samples at pool sizes of 30 samples; they claimed that this was probably because of the carrier effect of higher RNA content in pools [27]. However, we could not replicate this finding in our study, in which only two saliva samples in the pool of 30 showed reduction in Ct values, while others showed increase in Ct values or negative results when compared to the corresponding single samples tested by the LightMix E-gene kit. This could be due to random variation in pooling of small volume samples or RT-PCR, which was also noted by Lee et al. [28].

To date, only pooling of nasopharyngeal specimens or saliva RNA was reported [24–27,29], but we have demonstrated that pooling of saliva samples could be an acceptable alternative when pool sizes are small. The median Ct values of pooled NPS and saliva samples (in pool of 5 or 10) were reduced by 2–4 when compared to those of individual samples. Interestingly, for pools of 30, the median Ct values of pooled saliva were similar to those of pooled samples in pools of 5 or 10, while those of pooled NPS was reduced by >7 when compared to individual NPS samples. No significant difference in the detection rates between the pooled NPS and saliva samples was noted in the pool of 5, in which 100% of pooled saliva samples were tested positive by the Hecin test kit. In a previous study by Pasomsub et al. [29], pooling of saliva RNA showed good performance in SARS-CoV-2 detection, further investigation is warranted to determine if sensitivity would be higher when testing the mixed nucleic acids.

5. Conclusions

In summary, we have validated the performance of a dual-target SARS-CoV-2 real-time RT-PCR assay and demonstrated its performance in the detection of SARS-CoV-2 RNA in both pooled NPS and deep throat saliva samples.

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Declaration of interest

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Author contributions

CCY Yip and S Sridhar conceived the study design and drafted the paper. KH Leung and ACK Ng performed the experiment. CCY Yip, KH Chan, KKW To, JFW Chan, IFN Hung, VCC Cheng and S Sridhar were involved in analysis and interpretation of data. All authors agree to be accountable for all aspects of the work.

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