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Application of digital PCR to determine the reliability of Xpert Xpress SARS-CoV-2 assay with envelope (*E*) gene negative and nucleocapsid (*N2*) gene positive results

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ABSTRACT

This study used digital polymerase chain reaction (dPCR) to determine whether envelope (*E*) gene-negative and nucleocapsid (*N2*) gene-positive (*E-N+*) results obtained with the Cepheid Xpert Xpress SARS-CoV-2 assay are reliable. Using droplet digital PCR results as a reference, 18 of 22 *E-N+* samples with a low viral load (81.8%) were identified as true positives.

1. Introduction

In Cepheid Xpert Xpress SARS-CoV-2 (Xpert Xpress) assay, the dual targets of the envelope (*E*) and nucleocapsid (*N2*) genes are amplified. The result is interpreted as positive if either both genes or *N2* gene alone is detected [1]. *E-N+* results account for 3.2% of the positive SARS-CoV-2 samples tested in our center and similar rate has been reported by Khoshchehreh *et al.* [2]. Falasca *et al.* showed that centrifugation to concentrate the Xpert Xpress *E-N+* samples by 10 fold, followed by a repeated test, gave a negative result [3]. It is, therefore, recommended that *E-N+* results should be interpreted with caution as they may be false positives [3].

Droplet digital PCR (ddPCR) has superior analytical sensitivity, especially for the detection of SARS-CoV-2 in samples with a low viral load [4–10]. There are 2 types of digital PCR (dPCR) platforms: droplet-based (e.g., Bio-Rad QX200 ddPCR system), which is well-established, and nanoplate-based (e.g., Qiagen QIAcuity One system), which is new to the market.

This study aimed to use dPCR to determine whether *E-N+* results obtained with the Xpert Xpress assay are reliable.

Twenty-two respiratory samples collected from 19 patients with a previous *E-N+* result (Ct values ranging from 37.7 to 44.8) from the Xpert Xpress assay were included in the study. The samples were stored at -80°C for 3 months before retrieval for dPCR. 200 μL of samples was used for RNA extraction and eluted in 50 μL as previously described [11]. To assist in the determination of infection status, testing for IgG antibodies against SARS-CoV-2 nucleocapsid protein (anti-NP IgG) was performed on serum samples, if available, using the Alinity i qualitative assay (Abbott, Sligo, Ireland).

2. Cepheid Xpert Xpress SARS-CoV-2 assay

Samples were analyzed according to the manufacturer's recommendations. The limit of detection (LoD) claimed is 250 copies/mL.

3. Bio-Rad QX200 ddPCR

The ddPCR test on the QX200 system (Bio-Rad Laboratories, CA, USA) was performed according to the manufacturer's recommendations. All

Abbreviations: dPCR, digital polymerase chain reaction; ddPCR, droplet digital polymerase chain reaction; N gene, nucleocapsid gene; E gene, envelope gene; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; COVID-19, coronavirus disease 2019; LoD, limit of detection; PPA, positive percent agreement; NPA, negative percent agreement

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Table 1
Detection of SARS-CoV-2 by digital PCR in 22 samples with a Xpert Xpress SARS-CoV-2 result of *E-N+*.

Sample no.	Digital PCR (copies/mL)		RT-PCR	Sample type for digital PCR/RT-PCR, time of collection from symptom onset	SARS-CoV-2 IgG anti-NP antibodies, time of collection from symptom onset
	Qiagen QIAcuity One dPCR	Bio-Rad QX200 ddPCR	Xpert Xpress*E, N2* gene Ct		
1	47	271	Not detected, 39.8	NPS+TS, 25 days	Positive, 16 days
2	435	180	Not detected, 38.2	DTS, 35 days	Positive, 23 days
3	Not detected	70	Not detected, 42.1	DTS, asymptomatic	Positive, asymptomatic
4	Not detected	177	Not detected, 44.4	NPS+TS, 2 days	Not performed
5	495	744	Not detected, 37.7	DTS, 23 days	Positive, 13 days
6	50	No positive droplet detected	Not detected, 44.8	TA, 32 days	Positive, 6 days
7	52	832	Not detected, 40.5	DTS, 3 days	Not performed
8	Not detected	118	Not detected, 43.1	DTS, 33 days	Positive, 40 days
9	Not detected	172	Not detected, 43.9	DTS, 2 days	Not performed
10[a]	199	529	Not detected, 43.2	NPS+TS, asymptomatic	Positive, asymptomatic
11[a]	51	61	Not detected, 43.2	DTS, asymptomatic	
12[a]	49	1872	Not detected, 42.0	NPS+TS, asymptomatic	
13	50	85	Not detected, 41.4	DTS, 2 days	Positive, 14 days
14	Not detected	No positive droplet detected	Not detected, 40.7	NPS+TS, 6 days	Not performed
15[b]	Not detected	No positive droplet detected	Not detected, 43.7	DTS, 19 days	Positive, 8 days
16[b]	50	141	Not detected, 43.3	NPA+TS, 19 days	
17	53	137	Not detected, 43.2	Sputum, 11 days	Positive, 8 days
18	102	138	Not detected, 42.3	NPS+TS, 16 days	Not performed
19	Not detected	No positive droplet detected	Not detected, 43.1	DTS, 2 days	Not performed
20	300	650	Not detected, 39.0	NPS+TS, 210 days, reinfection	Positive, 210 days, reinfection
21	236	480	Not detected, 41.1	DTS, asymptomatic	Positive, asymptomatic
22	336	140	Not detected, 39.2	DTS, 22 days	Positive, 12 days

NPS+TS = Combined swabs collected from nasopharyngeal and throat; DTS = deep throat saliva (also known as posterior oropharyngeal saliva); TA = tracheal aspirate.
[a] Samples 10-12 were collected from the same patient for disease monitoring.
[b] Samples 15 and 16 were collected from the same patient for disease monitoring.

samples were tested in triplicate. The results were analyzed by using QX Manager, standard edition. Samples were interpreted as positive if ≥1 droplets were detected for either one (*N1* or *N2*) or both targets and confirmed by comparing with the positive control. The LoD claimed for the Bio-Rad SARS-CoV-2 ddPCR kit is 150 copies/mL.

4. Qiagen QIAcuity One

The Qiagen SARS-CoV-2 N1+N2 assay (Qiagen, Hilden, Germany) was performed using the Nanoplate 26k 24-Well protocol according to the manufacturer's recommendations. All samples were tested in triplicate, and results were analyzed by QIAcuity One system. The LoD claimed is 250 copies/mL.

Using QX200 ddPCR assay as a reference method, 18 of 22 samples (81.8%), with droplet concentrations ranging from 61 to 1872 copies/mL, were confirmed as positive, while 15 samples (68.2%) were detected with the QIAcuity One assay (Table 1). It showed that *E-N+* results generated with the Xpert Xpress assay were mostly true positives. The positive percentage agreement between the 2 dPCR platforms was 77.78% (Table 2). For the 18 true positive samples confirmed by ddPCR, the Ct values of the *N2* gene ranged from 37.7 to 44.4, and 16 of the Ct values were greater than or equal to 39. Of the 4 samples with no positive droplet detected by ddPCR, sample 6 tested positive using the QIAcuity One assay, with a concentration of 50 copies/mL. This was below the LoD of the QX200 ddPCR assay. For the 3 true negative samples (false positives with the Xpert Xpress assay) that were negative by both dPCR assays, the Ct values of the *N2* gene ranged from 40.7 to 43.7.

Discordant results for the 2 dPCR assays were observed in sample no. 3, 4, 6, 8 and 9. They had viral loads below or close to the LoD. We verified the analytical sensitivity of both dPCR assays using purified intact SARS-CoV-2 viral particles (NATtrol SARS-Related Coronavirus 2; ZeptoMetrix, NY, USA) and determined that the LoDs were 100 copies/mL. Further testing of 5 external quality assurance samples showed 100% concordance with the intended qualitative results, with a similar viral load detected by both platforms (Table 3). This provided further evidence that discrepancies occur mainly in samples with viral loads close to the LoD.

There was no positive droplet being detected by QX200 ddPCR on 2 samples (no. 6 & 15) and 3 samples (no. 3, 8 &15) were not detected by QIAcuity One assay, while these patients were seropositive that indicates a prior COVID-19 infection. Sample no. 15 & 16 were collected from the same patient on the same day and discrepancies in dPCR results was probably associated with sample quality as no. 15 was a saliva sample that self-collected by patient while no. 16 was a nasopharyngeal sample that collected by healthcare workers. These patients were likely have resolving infections with prolonged viral RNA shedding in their respiratory samples. As Hong Kong has adopted a Zero-COVID strategy, clinically stable patients will be monitored for the presence of SARS-CoV-2 before discharge.

Our findings suggested majority of *E-N+* results obtained with the Xpert Xpress assay were true positives, which is discrepant to the results reported by Falasca *et al.* [3]. It may due to different approaches used for the confirmation of such weakly positive results. In our laboratory, any sample with a Ct value ≥35 is confirmed using another RT-PCR platform, and a positive result is only reported if

Table 2
Comparison between the Bio-Rad QX200 ddPCR and the Qiagen QIAcuity 1 dPCR results for 22 samples that gave an *E-N+* result with the Xpert Xpress SARS-CoV-2 assay.

		Bio-Rad QX200 ddPCR		(95% CI)	
		Detected	Not detected	PPA	NPA
Qiagen QIAcuity One dPCR	Detected	14	1	77.78% (52.36 to 93.59%)	75.00% (19.41 to 99.37%)
	Not detected	4	3		

PPA = positive percent agreement; NPA = negative percent agreement; dPCR = digital polymerase chain reaction; ddPCR = droplet digital polymerase chain reaction; CI = confidence interval.

Table 3
Comparison of 5 QCMD EQAP SARS-CoV-2 samples analyzed with the Bio-Rad QX200 ddPCR and the Qiagen QIAcuity 1 dPCR assays.

Sample no.	Qiagen QIAcuity One dPCR log10 copies/mL	Bio-Rad QX200 ddPCR log10 copies/mL	QCMD EQAP (for reference only) log10 copies/mL
SCV2_21C1B-01	4.77	4.96	4.13
SCV2_21C1B-02	3.48	3.46	2.51
SCV2_21C1B-03	2.82	2.95	2.00
SCV2_21C1B-04	3.70	3.84	2.94
SCV2_21C1B-05	3.86	3.99	3.15

QCMD = Quality Control for Molecular Diagnostics; EQAP = external quality assurance program.

SARS-CoV-2 RNA is detected by both platforms. Otherwise, the result is reported as indeterminate. Clinicians are strongly advised to collect another sample (preferably a lower respiratory tract specimen) from patients with indeterminate results and manage them as presumptive positive while recommending repeat sampling. Testing for the presence of Anti-NP IgG could also be considered to investigate the possible false positive RT-PCR result.

Stoichiometric variability may also have contributed to the discrepancies between the 2 dPCR assays. The input volume of RNA was different, with 5.5 μL used for each of the 22 μL reactions in the QX200 ddPCR assay and 10 μL used for each of the 40 μL reactions in the QIAcuity One assay. Such a difference in the volume of template added may affect the amount of viral RNA available for the RT-PCR reaction.

In conclusion, our study showed that most of the *E-N+* Xpert Xpress results were true positives. There are several limitations of this study. First, the sample size was small. Second, dPCR was performed retrospectively and not a "head-to-head" comparison with the Xpert Xpress assay, which may have introduced bias into the analysis. Third, RNA may be degraded after prolonged storage or multiple freeze and/or thaw processes. Further prospective studies are therefore recommended.

Authors' contribution

River Chun-Wai Wong: Conceptualization, Data curation, Formal analysis, Validation, Investigation, Writing-original draft, Writing-review & editing. Ann Han Wong: Writing-review & editing. Yolanda Iok-Ieng Ho: Writing-review & editing. Gilman Kit-Hang Siu: Supervision, Writing-review & editing. Lam-Kwong Lee: Writing-review & editing. Eddie Chi-Man Leung: Writing-review & editing. Raymond Wai-Man Lai: Project administration, Supervision.

Declaration of competing interest

The authors declare that we have no conflicts of interest.

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Supplementary materials

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