Inconclusive results of SARS-CoV2 RT-qPCR: To retest or not?

Abstract

**Background:** Reverse transcription-quantitative PCR (RT-qPCR) is widely used to detect SARS-CoV-2 infections. A small proportion (3-5%) of the samples turn out to be inconclusive which are difficult to interpret and require repeat testing.

**Methods:** This study utilizing RT-qPCR for SARS-CoV-2 collected data from the viral RNA extracted using Maccura Mag-Bind RNA from NPOP specimen, then amplified and quantified using Maccura SARS-CoV-2 Fluorescent PCR kit. The data with inconclusive interpretation and re-test results were selected and further analysed.

**Results:** The retrospective analysis of 247 inconclusive samples that were retested was included in the study. Among the inconclusive results from the first test, 80% of samples which expressed SARS-CoV-2 N and E genes (without ORF1ab gene) turned out to be positive in the repeat test (p < 0.001), while 55% of samples that had only one gene expressed initially, were positive on repeat testing. The E gene was detected (without N and ORF1ab gene) in nine samples, of which seven were negative on re-testing.

**Conclusions:** Our study suggests that it is beneficial to repeat the SARS-CoV-2 RT-qPCR test, especially when two genes are expressed, while detection of only E gene in the first test can be regarded as negative.

**Keywords**
Inconclusive, Possibly Positive, RT-qPCR, COVID-19, SARS-CoV-2
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COVID-19, caused by the SARS-CoV-2 virus, was first isolated from an unexplained pneumonia case in 2019 in Wuhan, China, with the first case confirmed in Indonesia on 2 March 2020. COVID-19 has been fatal to a large number of patients worldwide, prompting the World Health Organization (WHO) to declare it as a pandemic on 11 March, 2020. The nucleic acid amplification test (NAAT) is currently the gold standard for SARS-CoV-2 detection, particularly reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR with multiple targeted genes such as open reading frame 1ab (ORF1ab), nucleocapsid (N), and envelope (E) are commonly used for detection. The detection sensitivity of RT-qPCR ranges from 32 to 95% in clinical samples, depending on the specimen type being processed.

Clear-cut positive or negative results are easy to interpret, whereas a small portion of samples (3-5% of all samples) with either only one gene detected or those with borderline cycle threshold (CT) values for any one of the genes are considered inconclusive (also referred to as “possibly positive” results). Several factors lead to inconclusive results, including inadequate sampling, a cold chain transport decline, poor viral transport medium (VTM) quality, insufficient sample storage, lack of amplification in internal control, or extraction failure. Inconclusive results are difficult to interpret and necessitate repeat testing, which increases laboratory cost, requires additional manpower, and delays reporting of result to the hospital or patient. Nonetheless, retesting of inconclusive RT-qPCR results is required for confirmation and decision-making regarding patient management strategy. Inconclusive cases might indicate the initial or late stage of COVID-19 infection which require a different patient management strategy, including isolation and testing approach. This study aims to estimate the value of retesting the inconclusive results and finding a better method of interpreting them in the first testing round itself, thereby reducing the need for unnecessary re-testing.

Methods
This study used data from samples tested for SARS-CoV-2 by RT-qPCR at the COVID-19 Laboratory, Atma Jaya Catholic University of Indonesia, from September to November 2020. A retrospective study of the 14,609 SARS-CoV-2 RT-qPCR results during this period was carried. The study was approved by the Institutional Review Board of the School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia (AJCUI) under the approval number 07/06/KEP-FKIKUAJ/2022. This study was a retrospective study, which required no further consent from participants and has been waived by ethical committee. Viral RNA from nasopharyngeal and oropharyngeal swabs was extracted using Maccura Mag-Bind RNA (Maccura Biotechnology Co., Ltd., Chengdu, PRC) based on the magnetic-bead principle, and further amplified using a qRT-PCR kit (SARS-CoV-2 Fluorescent PCR, Maccura Biotechnology Co., Ltd., Chengdu, PRC) to detect ORF1ab gene (FAM), N gene (Cy5), and E gene (ROX) with Internal Control/IC (VIC) on CFX 96 Touch Thermal Cycler (Bio-Rad Laboratories). The amplification was done as per manufacturer’s instruction, with cycling conditions of 55°C for 15 minutes, 95°C for two minutes, followed by 40 cycles at 95°C for 15 seconds, 58°C for 35 seconds, and a final cycle at 40°C for 10 seconds. Through a quality control assessment, valid results were amplified and checked for whether they showed a typical smooth “S-shaped” curve above the threshold. The CT value results were analysed using Bio-Rad CFX Maestro software (V4.1.2433.1219). Based on the CT value analysis, a retest was conducted on the inconclusive samples using the same extraction and PCR kits used in the first round.

The positivity cut-off of the CT value was decided as less than or equal to 38 for both N and ORF1ab genes and less than or equal to 37 for E gene. The sample was categorized as positive if the ORF1ab gene was detected along with the N gene and/or E gene (based on the CT cut off). Negative result was confirmed if no genes were detected. Inconclusive results were classified into four groups based on gene(s) detected (as specified by the manufacturer): i) ORF1ab gene only (ORF1ab+), ii) N gene only (N+), iii) E gene only (E+), iv) both N and E (N+E+) gene detected but without ORF1ab gene. The inconclusive results were retested and categorized as positive if the targeted genes were detected within the CT cut-off value and negative if no gene was detected.

Statistical analyses were performed using STATA version 14.1. Among the inconclusive group, frequencies and CT values of each gene from the first test were compared with the retest results (positive or negative) using the independent t-test and Chi-square test. Regarding the frequency of inconclusive groups, N+ and ORF1ab+ were compared using chi-square test and the reference was N+E+ group, whereas E+ group was compared using Fisher’s exact probability test because chi-square test assumptions were not met. The CT value of each gene in the inconclusive test was compared using the Mann-Whitney U test for all genes, except N gene of the N+E+ group which was compared using unpaired t-test as it showed normal distribution. CT value of each gene from the same sample was compared between the first test and retest using paired t-test for N gene of N+E+ group, while CT value of other genes were calculated using a Wilcoxon signed-rank test, because the distribution was not normal. A p-value less than 0.05 was defined as statistically significant.
Results and discussion

A total of 14,609 samples were processed using the Maccura Mag-Bind RNA viral RNA extraction kit and consecutively amplified using the Maccura SARS-CoV-2 Fluorescent PCR kit. Of the total samples tested, 11,220 (76.8%) samples were negative with 17.26% (2521) being positive, and 4.25% (621) of samples having invalid results, which were retested and further classified as positive, negative or inconclusive. A total of 247 samples were recorded as inconclusive, representing 1.69% of the total samples analysed (Figure 1a) which is much less than previously reported (4-5%). Inconclusive cases can be minimized, though unavoidable, as a small portion will always occur. Inconclusive results might happen due to aberrancies at multiple levels in the process of retesting; therefore, it is vital to evaluate all processes including sample collection, quality of VTM, sample transportation, sample storage prior to processing, RNA extraction or PCR process.

The stratification of the inconclusive groups in our study is represented in Figure 1b. The majority (65.59%) of the inconclusive samples were N+ (162), followed by ORF1ab+ and N+E+ inconclusive categories with 18.62% (46) and 12.15% (30), respectively. E+ contributes to nine samples (3.64%). The retesting of these inconclusive samples cleared 122 samples (49.39%) as positive and 126 samples (50.61%) as negative which is comparable with previous reports, in which 53.9% of inconclusive samples turned positive on repeat testing. Although false-negative results of SARS-CoV-2 RT-PCR are predicted to be around 28% to 39%, our study found that 126 out of 247 samples (51%) were false negative results.

This study also showed that when two genes (N+E+) were detected, the probability of positive result on retest was significantly higher (80%) (p-value = 0.001) than when only one gene was detected (ORF1ab+, N+ or E+ alone) (45.16%). Table 1 represents significant differences between N+E+ samples as compared to N+ only (p-value = 0.001), ORF1ab+ only (p-value = 0.002), and E+ (p-value = 0.003). Moreover, we observed that E+ in the first test had the least chance of turning positive on repeat testing. This observation could be explained by the fact that the E gene has specificity problems and is vulnerable to sample contamination. Overall, our data indicated that retesting inconclusive samples was beneficial, particularly when two genes were detected, but was ineffective when only E gene was detected.

We investigated whether the CT values of inconclusive samples correlated with retested positive results. We observed that the CT value of the inconclusive samples could not predict the positivity following a retest since there were no significant differences between CT values of the inconclusive first test and positive retest result, as presented in Table 2. One possible explanation could be that the cut-off CT value is higher than 37, which correlates with a lower RNA load; a major difference in borderline CT values was not observed in the positive versus the negative final result of the inconclusive samples tested.

![Figure 1. Samples distribution.](image)

**Figure 1.** Samples distribution. (a) Pie chart representing the total selected samples which consist of negative (dark grey), positive (light grey), invalid (grey) and inconclusive (red) shown in percentage (%). (b) A bar chart showing the stratification of inconclusive groups (actual cases numbers).
We further selected samples paired with final positive results (first and second test) of all inconclusive groups and compared both CT values as summarized in Table 3. Another interesting observation was that the CT value of the positive retest was significantly lower compared to the first test CT value for inconclusive samples. This finding suggests the importance of retesting inconclusive results in order to improve diagnosis accuracy.

There were some potential aspects that could be assessed in the future research based on the results from our study. The lack of access to clinical and epidemiological data that could have aided in the analysis of this investigation was a major limitation. Further studies should consider incorporating these data to provide a more comprehensive understanding of the results. The use of larger sample sizes and diverse cohorts could also enhance the generalizability of the findings. Additionally, integrating patient characteristics such as age, sex, and co-morbidities could help in understanding the impact of these factors on test outcomes. Future research could also explore the role of retesting in reducing diagnostic uncertainty and improving clinical decision-making.
fallback. Additionally, this study used only one extraction kit and one PCR kit, contributing to consistent results. However, the inconclusive results might vary when using other kits. Further studies could be conducted to compare extraction and PCR kits, analyze the CT value in greater detail, or compare the results to those from another country or laboratory. In conclusion, most inconclusive samples expressed only the N gene, followed by only the ORF1ab gene, and both N and E genes together. When two genes were expressed in the first test, 80% of the inconclusive samples tested positive (p = 0.001) on the retest, compared to samples with only one gene expressed (45%). Detection of the E gene alone was found in nine samples, of which seven were negative in the retest. Retesting the inconclusive samples was beneficial, especially when two genes were expressed, but there was not much value in retesting when only the E gene was expressed. Altogether, our data might become a reference for similar investigations done in multiplexing targets of other pathogens.\textsuperscript{15}

Data availability

Underlying data

Figshare: Dataset_Inconclusive RT-qPCR_MK, https://doi.org/10.6084/m9.figshare.20306415v1.\textsuperscript{16}

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

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References

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