Reliability of RT-qPCR Pooling Method for COVID-19 Detection in Various Cycle Threshold Values

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Abstract

Background: Reverse transcriptase quantitative real-time polymerase chain reaction (RT-qPCR) is a standard method to detect SARS-CoV-2, the cause of COVID-19 disease, albeit expensive for some laboratory settings. The pooling test is widely used for large-scale screening to speed up the turn-around time and reduce the cost of the RT-qPCR. However, the pooling test involves mixing a certain number of specimens which theoretically increases the possibility of false-negative results. This study aimed to evaluate the accuracy of the pooling test compared with the non-pooling test in different Ct values as a surrogate for viral load.

Methods: RT-qPCR was performed in three groups of samples: non-pooling (individual samples), pooling of 5 samples and 11 samples, with various ranges of Ct value in the respective group: x<25 (n=4); 25<x<30 (n=5), x<30 (n=16), and negative sample (n=5). Agreement and kappa values were calculated. Four of twenty-five individual samples resulted in false-negative after pooling.

Results: By taking all samples without applying the cut-off value to the calculation, the agreement in pooling of 5 samples was 0.86 (Kappa 0.31) and of 11 samples was 0.64 (Kappa 0.96). When the cut-off value of Ct<37 was applied, percent agreement and kappa were 1.00, respectively, for both pooling methods.

Conclusions: Pooling up to 11 samples shows high concordance with RT-qPCR with individual samples with Ct<37. Interpreting pooling results in a very low viral load (Ct≥37) must be considered due to the increased possibility of inconclusive results.

Keywords: Cycle threshold (Ct) value, COVID-19, pooling test, RT-qPCR

Introduction

Coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which first appeared in Wuhan City, China, in late December 2019. SARS-CoV-2 is believed to be part of an animal coronavirus that mutates, spreads and rapidly evolves among the human population.[1] SARS-CoV-2 can be transmitted through feces, blood, contaminated object, but the direct transmission is via respiratory droplets,[2] therefore, a quick investigation and sufficient tools to contain the pandemic and break the chains of transmission is needed. The clinical spectrum of COVID-19 disease is heterogeneous, ranging from asymptomatic to organ damage with delayed diagnosis. Patients with COVID-19 can increase risk of infection for caregivers, as well as the mortality rate of patients.[3,4]

According to the World Health Organization (WHO), there are more than 444 million cases, with a death toll of more than five million cases until March 2022. Indonesia has more than five million positive cases, with more than 150,000 confirmed deaths.[5] Although COVID-19 transmission has weighed Indonesia's burden, the country has brought down the COVID-19 cases from its peak and has maintained this trend from September 2021 to the end of the year. However, it escalated again at the end of January 2022, when the Omicron variant emerged. This variant is revealed to be ten times more contagious compared to the original variant.[6] Globally, BA.5 variant of Omicron is spreading across several areas and proved to reduce neutralization titres in in

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vitro studies, in vaccinated and convalescent cohorts.[7]

To date, the most common method to detect infection of the SARS-CoV-2 virus is reverse transcriptase quantitative real-time polymerase chain reaction (RT-qPCR), which is relatively expensive for resource-limited countries.[8,9] While efficiency in handling COVID-19 is urgently needed, community transmissions are still difficult to control, and extensive contact tracing should be supported by accurate and sensitive population scale testing.[10,11] Therefore, many countries have applied a pooling test for extensive scale screening to reduce costs and speed up the diagnostic process.[12]

A pooling method involves mixing several samples in a tube before performing the test. When the test result of the group is negative, all individuals in the respective pool can be declared as negative. In contrast, a positive result indicates that one or more individuals in the group have positive results. In this case, re-testing of individual specimens will be carried out.[13] Several factors can affect the pooling test's accuracy, including the pool size and disease prevalence.[14] Unfortunately, published data from Indonesia regarding pooling test accuracy in various pool sizes and Ct values is still limited.

This study aimed to evaluate the reliability of the RT-qPCR pooling method in various cycle threshold (Ct) values compared to regular RT-qPCR covid testing protocol. Then, to ensure that the pooling test might help to detect low to high Ct values, positive samples with various Ct values were included in this study. The pooling of five and 11 samples were evaluated. Each pooling test was compared with RT-qPCR from the individual sample (non-pooling method). The agreement between pooling and individual samples was calculated.

Methods

Samples were selected from the collection (bio-archive) of specimens in the Biosafety Level 3 (BSL-3) facility, Central Laboratory, Universitas Padjadjaran, as one of the National laboratory networks for COVID-19 (Code C.67) as recommended by the Ministry of Health at HK.01.07/MENKES/4642/2021. The selected Ct value of individual SARS-CoV-2 positive samples spanned a large range of Ct of lower than 40 and was selected randomly by considering viral transport medium (VTM) condition. VTM lower than 300 mL, change in color, and inactive VTM type would be excluded. The samples included different Ct values: x<25 (n=4); 25<x<30 (n=5), x>30 (n=16),

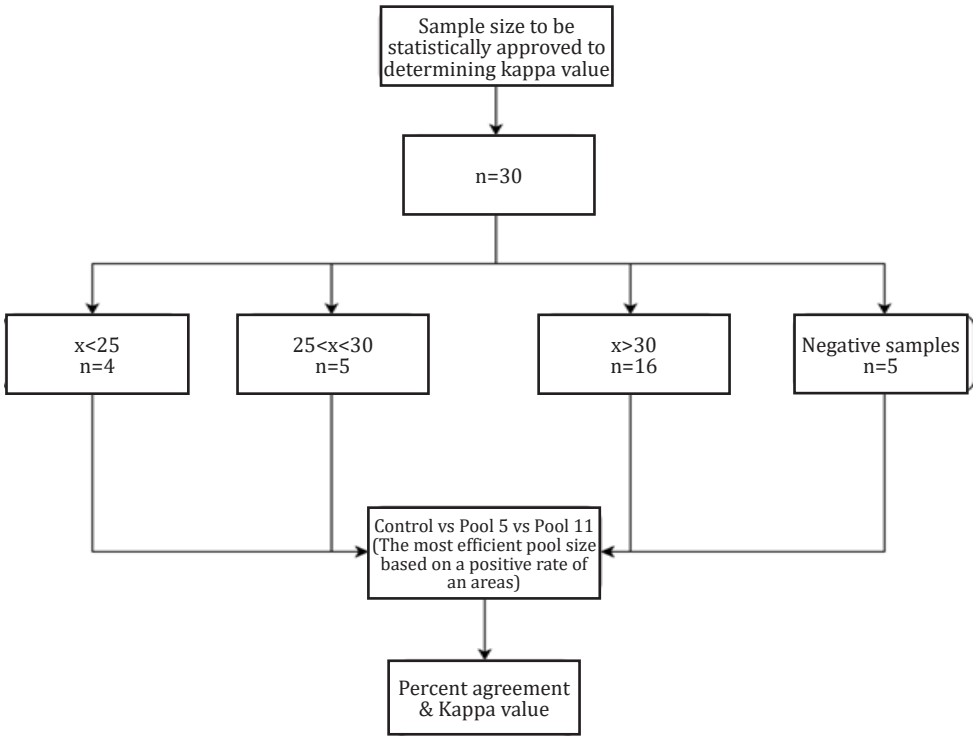

Figure 1 Research Flowchart

Table 1 Cycle Threshold Values Non-Pooling vs. Pool 5 vs. Pool 11

Subject	Non-Pooling			Pool 5			Pool 11		
	N Gene	orf1ab Gene	Internal Control Gene	N Gene	orf1ab Gene	Internal Control Gene	N Gene	orf1ab Gene	Internal Control Gene
A	37.86	38*	23.74	40.86*	40.7*	26.41	41,36*	41.5*	28.19
B	21.13	21.66	24.36	23.52	24.63	26.86	24.72	25.92	27.49
C	16.16	17.29	23.96	19.48	20.51	27.91	19.87	20.92	27.95
D	30.02	30.01	28.51	31.94	32.68	31.29	33.59	34.65	32.32
E	28.87	29.85	28.45	30.33	30.85	29.8	31.4	32.08	31.56
F	20.67	21.57	25.76	23.02	24.13	28.25	24.31	25.39	29.3
G	33.49	33.5	23.21	36.08	36.77	27.07	37.22	37.58	28.57
H	26.55	26.61	26.94	29.87	30	29.41	31.14	31.47	30.81
I	35.01	35.5	30.02	38.52	38.53	33.78	38.29	38,99*	32.12
J	28.82	29.74	28.37	30.36	31.19	30.25	30.83	32.1	30.88
K	20.77	21.62	25.93	23.66	24.78	28.75	24.71	25.89	29.92
L	34.56	34.84	24.22	36.61	36.87	26.53	38,06*	38.33	27.83
M	33.46	34.06	24.72	36.58	36.33	28.04	36.32	36.02	27.6
N	34.27	34.08	23.27	34.73	36.12	25.98	38.02	37.39	27.26
O	35.19	35.36	25.04	38.39	37.69	26.95	38,69*	38.11	28.61
P	33.46	34.06	24.72	36.58	36.33	28.04	36.32	36.02	27.02
Q	34.27	34.08	23.27	34.73	36.12	25.98	38.02	37.39	27.26
R	35.19	35.36	25.04	38.39	37.69	26.95	38,69*	38.11	28.61
S	36.71	36.07	19.02	36.83	38.42	23.57	40.33*	36.79	22.39
T	36.59	36.17	20.48	38.58	38.18	23.57	40.09*	38.48	23.91
U	37.47	38*	22.4	40.47*	41	28.79	40.97*	41.5*	29.46
V	37.17	38.23	23.28	40.17*	41.23*	26.92	40.87*	41.93*	27.66
W	38*	37.05	23.95	41*	30.05*	28.1	42*	40.75*	28.96
X	23.69	23.86	19.95	26.51	26.88	24.25	35.41	34.59	20.04
Y	28.99	29.01	25.5	36.77	38.23	24.02	40	38.08	26.46

Note: *Retested or adjusted above 40 as gene detected (-)

and negative samples (n=5). Specimens with Ct values below 25 were considered to have a high RNA viral load, while those with Ct values between 25 and 30 were considered to have an intermediate viral load. Specimens with Ct values higher than 30 were considered low viral load, thus representing culturable virus amounts, and have been assumed to be infectious.[15] Pools of five & 11 (n = 30 each) were made to be compared to non-pooling RT-qPCR (n=30) (Figure 1).

The minimum sample size to calculate the kappa value and the most efficient pool size based on the positivity rate of an area were considered before conducting the study. Considering that the pool testing was useful for populations with infection rates below 5% and our goal was to cut the usual cost of RT-qPCR pooling five & 11, a total of 30 specimens from the Central Laboratory Universitas Padjadjaran were taken for our study.[16,17]

This study simulated a pooling test conducted in low prevalence conditions and low resource settings. To obtain five and 11 pooling samples, fifty microliters of the positive specimen, acting as one positive sample, were mixed with 200 and 500 µL of VTM (brand Citoswab and iblue) simulating four and ten negative samples. After that, 200 µL of non-pooling samples and 200 µL of pooled samples

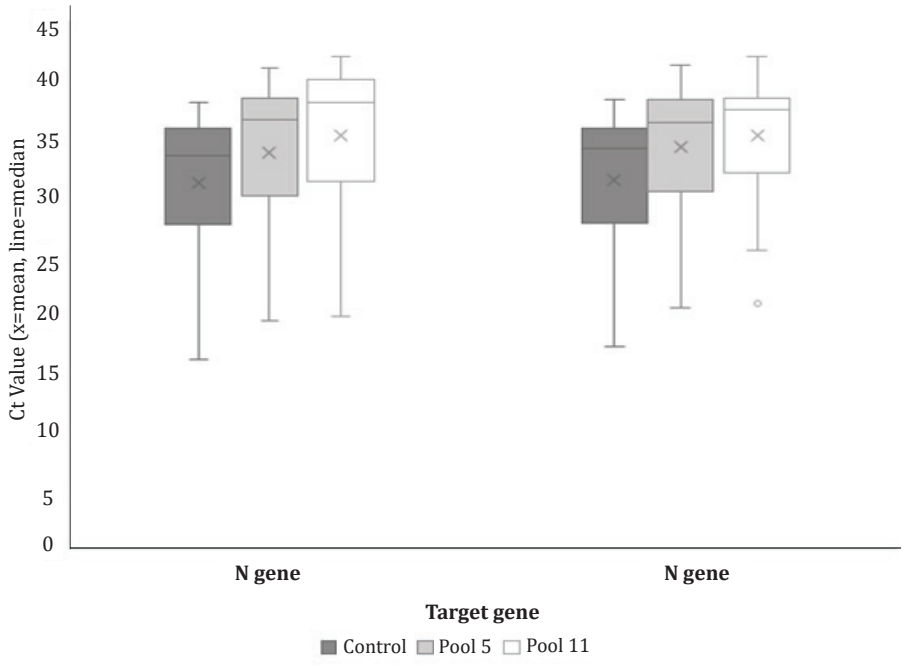

Figure 2 Average of Cycle Threshold Value Samples with Various Treatments

from the mixture were extracted using the Guangzhou DaTe SARSCov-2 extraction kit. 75 µL RNA acquired from the extraction were amplified using DaAn Gene SARSCov-2 RT-qPCR Kit on LightCycler® 96 System. DaAn Gene kit employs two one-step RT-qPCR assays using fluorescent probes for alternative SARS-CoV-2 genes, called ORF1b and the N gene and endogenous internal control. The Ct value and the mean cycle thresholds difference (Δ Ct) of N and ORF1ab gene were also analyzed to see the effect of dilution on the sample in the pooling test.

This study obtained Ethical Approval issued by the Research Ethics Committee of Universitas Padjadjaran with No. 168/UN6. KEP/EC/2021.

The RT-qPCR results were interpreted by applying Lightcycler 96 SW 1.1, and the determination of the positive and negative samples was based on the instructions in the DaAn kit manual. If the test sample had no amplification curve or Ct>40 in the channel but has amplification in the internal control channel, the sample could be judged as negative. The sample could be considered positive if all the genes appeared and Ct was not more than forty. If only one of the genes had amplification, it was recommended to repeat the test. If the retest was consistent with the previous result, it was reported as positive; however, if the retest was negative, it was reported as negative.

Several samples with the same or different results between pooling and non-pooling entered into percent agreement and Cohen's kappa calculation to obtain information about the similarity and reliability of the pooling method compared to the RT-qPCR non-pooling method.[18]

Results

The various cycle threshold values of the experiments by pool size and target gene was shown in Table 1. Four samples out of 25 non-pooling positive samples had a negative value when entering pooling five and pooling 11. Several inconclusive results in pooling 11 turned positive after retesting according to DaAn gene manual instructions (Table 1). The average of cycle threshold value samples with various treatments was shown in Figure 2.

Additionally, pooling samples generally had higher Ct values than those obtained from a single sample where the mean (X sign) in each treatment was 31.13, 33.72, and 35.25 for the N gene while for Orf1ab gene were 31.42, 34.24, and 35,2. The mean Ct difference (ΔCt) between a sample of the non-pooling with pool

Table 2 Two-by-two Table of Pool Method vs. Non-Pooling Results

		Non-Pooling Results		Total test N=30	Agreement (%)	Kappa Value (95% CI)
		Positive N=25	Negative N=5			
Pool 5	Positive	21	0	21	86	0.64 (0.31; 0.96)
	Negative	4	5	9		
Pool 11	Positive	21	0	21	86	0.64 (0.31; 0.96)
	Negative	4	5	9		

Table 3 Two-by-two Table of Pool Method vs. Non-Pooling Results (CT< 37)

		Non-Pooling Results		Total test N=26	Agreement (%)	Kappa Value (95% CI)
		Positive N=21	Negative N=5			
Pool 5	Positive	21	0	21	100	1
	Negative	0	5	5		
Pool 11	Positive	21	0	21	100	1
	Negative	0	5	5		

five and the non-pooling with pool 11 were 2.59 and 4.11 for the N gene, while the Orf1ab gene was 2.81 and 3.78 (Figure 2). These values helped us to see the effect of dilution on the sample in the pooling test and predicted the range detection of the SARS-CoV-2 Ct value that could be detected by the pooling five method up to the pooling 11 method RT-qPCR.

The percentage of agreement and Cohen's Kappa calculations were conducted as shown in Table 2 and Table 3.

Discussion

Contact tracing is a mandatory to prevent the massive transmission of SARS-CoV-2, however, proper tracing is hampered by the high-cost of RT-qPCR as the gold standard test, and the need for rapid turn-around time diagnosis. The pooling method is one of the alternative solutions for large-scale screening and contact tracing. However, different pooling methods are applied in different settings, and reports of the reliability of pooling method are still limited. This study evaluated pooling of five and 11 samples and calculated the agreement (and Cohen's Kappa) compared with the non-pooling (individual) sample results. The results showed that the agreement of both pooling was excellent for samples with Ct<37 compared with the non-pooling method.

Other studies have shown that pooling five to 11 samples is recommended and acceptable; although there are shifting Ct values in the pooling method, this technique is considerably reliable.[19–21] Theoretically, the Ct value shifts -3.3 in 100% qPCR efficiency in 10-1 dilution.[22] As the increase of pool size, the shifting in Ct values follows a linear regression model: Difference Ct=0.187×pool size (n)+0.498 (R2=0.53). Dorfman equation shows that the optimal pool size is 11 when the disease prevalence is ±0.66%, as shown by a study in Korea.[23] Therefore, to model pooling test performance in low prevalence and low resource setting, pool size five and 11 was evaluated in this study.

Pooling of five and 11 samples still shows perfect agreement with RT-qPCR from the individual sample if the Ct<37 as in individual studies shows that pooling does not affect the sensitivity of detecting SARS-CoV-2 when the Ct of the original specimen is lower than 35 and performed in ten samples pool setting.[24,25] However, samples with Ct>37 could be negative or inconclusive when, included in pool 11, making a false negative result. On the other hand, some literature reported that the SARS-CoV-2 culture positivity rate decreases progressively on Ct value 33.[26] No viral cultures were obtained from samples with Ct>34.[27] The culture specimens were last detected at Ct values=35.[28] It was assumed that pooling samples to up to 11 in our experiment was still

reliable for implementation because Ct values >37 had a low viral load for a chain infection.

This study has only used one type of VTM and RT-PCR kit in the pooling experiment, this is a limitation of this study. Indonesian laboratories use various types of VTM, including inactive VTM as some compositions may disturb the RNA extraction process and molecular detection result.[29] Moreover, various RT-PCR kits, ranging from simplex to multiplex kits were also used in Indonesian clinical laboratories—possibly having various Limits of Detection (LoD). Lastly, a community-based study is also needed to evaluate the implementation of pooling five and 11 samples with various disease prevalence ranges.

In conclusion, pooling up to 11 samples has demonstrated high concordance with RT-qPCR with individual samples with Ct<37. Interpreting pooled results at very low viral loads (Ct≥37) should be considered due to the increased possibility of inconclusive results.

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