Short Report

Evaluation of sample pooling method for SARS CoV-2 RNA detection; a laboratory based simulated study

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Abstract

Real time RT-PCR is considered as the gold standard test to detect COVID-19. The use of sample pooling strategy increases testing capacity and spares resources. However, the effectiveness of sample pooling should be evaluated in the setting before being implemented. Forty five samples including 20 high positives (Ct<20), 20 low positives (Ct 20-40) and 05 negative samples were used to prepare 1:1, 1:3 and 1:5 simulated sample pools which were then subjected to viral RNA extraction followed by real time RT-PCR. Sensitivity and specificity of sample pooling technique in the detection of SARS-CoV-2 RNA was 100% without significant variation of Ct values. According to our results, pooling of up to 6 samples will not have an effect on the final result in clinical samples and hence can be adopted in the given context for the diagnosis of COVID-19 by RT-PCR.

Keywords: SARS CoV-2, Sample pooling, RT-PCR

Introduction

COVID-19 is caused by a newly discovered coronavirus, SARS-CoV-2.[1] Detection of COVID-19 in symptomatic patients and asymptomatic carriers, case isolation and contact tracing are important in controlling the spread of the infection.[1,2] It is mandatory to evaluate the reliability and accuracy of laboratory testing methods used in the diagnosis of COVID-19.

Real time RT-PCR is considered as the gold standard method in the diagnosis of COVID-19 and is widely used.[1] The prevailing situation of COVID-19 has demanded smart testing strategies while testing of pooled samples has become effective for mass screening of people, especially when the workload exceeds capacity and resources are limited. Different laboratories have adopted different techniques for which there are published protocols.[3] One such method is

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pooling of samples at the RNA extraction step, where multiple samples are mixed and viral RNA extracted as a single sample.[3-5] In this sample pooling strategy, false negative results could be obtained, and borderline positive samples will be missed due to the dilution effect. Although there are published protocols, laboratories should do their own validation and evaluation of cost effectiveness before implementing this strategy.[6]

We evaluated the use of sample pooling in extracting viral RNA in the local laboratory setting with regard to the sensitivity, specificity, and diagnostic accuracy.

Methods

Forty five nasopharyngeal swab samples collected in transport medium (Plastica, Sri Lanka) including 20 high positives (Ct<20), 20 low positives (Ct 20-40) and 05 negative samples were selected based on initial PCR results. Samples were collected from symptomatic patients and close contacts during September to November 2021 and stored at -80 ^{o}C with continuous temperature monitoring. Number of samples was based on guidelines on clinical and laboratory validation of diagnostic assays.[6] Samples were thawed and diluted serially using the same unused transport medium, to resemble pooling of multiple samples. A series of sample dilutions was prepared using transport medium making 1:1, 1:3 and 1:5 dilutions simulating pooled samples 2, 4 and 6 respectively in duplicates. Nucleic acid extraction was carried out from dilutions (resembling pooled samples) and neat samples by manual spin column method according to the manufacturer's instructions using a locally validated commercial kit (Promotor® DNA/RNA isolation kit, Germany). Extracted RNA was subjected to real time RT-PCR by using commercial PCR assay (Real Star, Altona, Germany) according to manufacturer's instructions. The same thermal cycler (Bio Rad CFX 96, USA) and pipette set was used during the entire experiment. Results were recorded with Ct values for each target gene, confirmatory gene (S gene) and screening gene (E gene) for SARS- CoV-2. Ct value 40 was considered as the cut-off of positive and negative based on the manufacturer's instructions. The sensitivity of pooled samples in each series was calculated. One-Way ANOVA test was used to calculate the significant difference between the dilution groups with the neat sample group.

Results

A total of 135 simulated pools of each dilution and 45 neat samples in duplicates were analysed. All positive individual samples were detected as positives in pooled samples at the tested dilutions of 1:1, 1:3 and 1:5. Sensitivity of sample pooling of up to six samples was 100%. Specificity was not affected, and it was 100% (Table 1).

In low positive samples, the mean Δ Ct shift was higher than that of high positive samples for S gene (mean Δ Ct = average Ct value of duplicates of each dilution – neat). However, significant variation was not observed in both high ($p = 0.399$) and low ($p = 0.489$) positive samples. An increased mean Δ Ct shift was observed in low positive samples ($p<0.05$) when compared to that of high positive samples ($p = 0.509$) for E gene. (Figures 1a and 1b).

Table 1: Mean Δ Ct Shift, Ct value range and diagnostic accuracy of each simulated pools

S gene detection					
High positive samples		Neat Sample	1:1 Dilution	1:3 Dilution	1:5 Dilution
	Ct value range	7.25-20.40	7.70-20.80	8.30-21.50	9.20-22.00
	Mean Δ Ct shift (±SD)	-	0.49 ±0.17	1.23 ±0.19	2.02 ±0.21
	Diagnostic accuracy		100%	100%	100%
Low positive samples	Ct value range	22.25-37.25	22.80-38.15	23.60-39.05	24.15-39.95
	Mean Δ Ct shift (±SD)	-	0.65 ±0.17	1.44 ±0.25	2.26 ±0.27
	Diagnostic accuracy		100%	100%	100%
E gene detection					
High positive samples		Neat Sample	1:1 Dilution	1:3 Dilution	1:5 Dilution
	Ct value range	7.35-19.20	7.80-19.60	8.45-20.85	9.35-21.05
	Mean Δ Ct shift (±SD)	-	0.47 ±0.18	1.29 ±0.19	2.10 ±0.14
	Diagnostic accuracy		100%	100%	100%
Low positive samples	Ct value range	20.30-37.15	20.75-38.15	21.40-39.05	21.95-39.85
	Mean Δ Ct shift (±SD)	-	0.65 ±0.18	1.47 ±2.43	2.29 ±0.30
	Diagnostic accuracy		100%	100%	100%

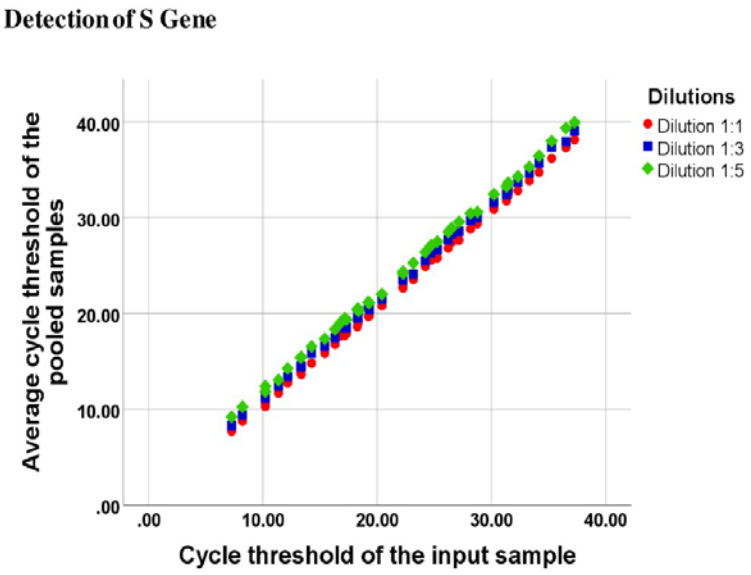

Figure 1a: Mean Δ Ct for each simulated pool

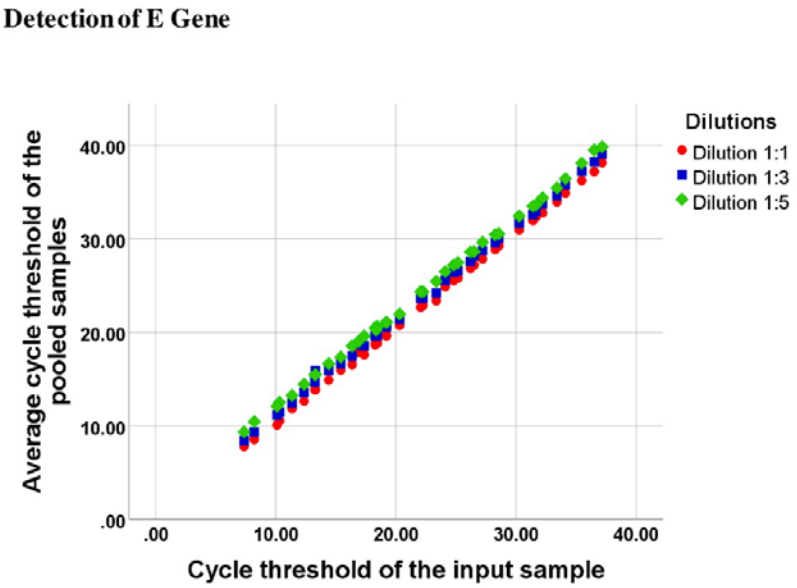

Figure 1b: Mean Δ Ct for each simulated pool

Discussion

The ability to detect viral RNA was not affected in pooling up to 6 samples in a single pool in our study. A study conducted to determine the optimal size of sample pooling for SARS CoV-2 RNA testing demonstrated that RNA positive samples with higher Ct values (>35) became negative when eight or more samples were pooled,[7] and sensitivity of a pool of six samples was 97.8%.[7] Salazar et al[8] demonstrated that the sensitivity and specificity of the pooling technique was 85.48% and 100% and positive and negative predictive value was 100% and 98.94% respectively. However, some studies showed lower sensitivities for 4-pooled samples (75%).[9] Our study obtained 100% sensitivity and specificity, and interestingly, both positive and negative predictive value was 100% when up to 6 samples were tested as a single pool. This difference could be due to differences in viral RNA amount in the original samples, or variations in the methods used for testing. There is a high chance of getting false negative results due to pooling of low positive samples. In addition, the present study was a simulated study and there was no effect of accumulation of inhibitors and other cellular content from different samples.

In our experiment, the average shift of Ct value was 2.0-2.2, when up to six samples were pooled, therefore shifting the cut-off value by +2 can be considered. However, this will create problems as some kits tend to give non-specific signals in late cycles.

The sample pooling technique is best suited for community screening. However, sample pooling should be done only after evaluating the prevalence of COVID-19 in the community.[10] When the prevalence of COVID-19 is high, adoption of pooling samples is not recommended as in such situations, the majority of pools will become positive necessitating deconvolution of sample pools and re-testing.

The results of this study provide resolutions for queries such as limiting the testing capacity, delay in issuing results, shortage of reagents and high turnaround time that arose during the COVID-19 pandemic. Pooling beyond 6 samples or more was not evaluated, considering practicality in the local laboratory setting. We also thought pooling of more samples will lead to more confusion and errors during procedures when using manual extraction. A logistical reason like capacity of consumable items used for pooling is a major limiting factor.

Conclusion

The results of this study provide preliminary data on pooling up to 6 samples processed as a single sample for the detection of COVID-19 by RT-PCR in a resource limited local laboratory context.

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PASLW – laboratory experiments, writing original draft; MARVM – supervision, review and editing; HMTUH – supervision, review and editing

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