Short Report

Assessing the effectiveness of sample and RNA extract pool testing for the detection of SARS-CoV-2 RNA by real time RT-PCR

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Abstract

Introduction: Pooled testing is a cost-effective approach to increasing testing capacity during pandemics. This study analysed the effectiveness of pooling of samples and RNA extracts for the detection of SARS-CoV-2 by real time RT-PCR.

Methods: Twenty SARS-CoV-2 positive samples with Ct value of 25–35 and 60 known negative samples based on initial PCR results were used for this study. The samples were used to prepare 2-, 4- and 8-fold pooled samples prior to extraction. The RNA extracts of a further 10 PCR positive samples were pooled to prepare 2-, 4- and 8-fold RNA extract pools. The Ct values of neat samples and pooled samples were compared using the paired *t*-test with a 95% confidence interval. The same was done for the neat RNA extracts and the extract pools.

Results: The detection capacity was considerably lower when the pool size was increased from 2- to 8-fold in sample pooling, whereas pools of RNA extracts showed 100% detection from 2- to 8-fold dilution. The increase in Ct value of 2-, 4- and 8-fold sample dilutions were 1.69 ± 0.78, 3.84 ± 1.47 and 8.98 ± 2.25, respectively ($P < 0.0001$). However, there was a small rise in the Ct value of 2-, 4- and 8-fold extract pools (1.01 ± 0.38, 2.18 ± 0.82 and 3.05 ± 0.77, respectively).

Conclusions: Large scale screening of asymptomatic individuals for SARS-CoV-2 can be maximised with optimal use of resources by 2- or 4-fold pooling of samples or 4- or 8-fold pooling of RNA extracts without significantly compromising the detection capacity.

Keywords: *Pooled testing, COVID-19 screening capacity, real time RT-PCR*

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Introduction

There was an increase in the laboratory testing load during the corona virus disease-19 (COVID-19) pandemic to meet the demand for testing.[1] Healthcare systems around the world, including Sri Lanka, increased their testing capacity to identify infectious cases and quarantine them, thereby containing the spread of the virus. Due to its high sensitivity and specificity, the gold standard assay for laboratory diagnosis of COVID-19 / severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection is the real time reverse-transcription polymerase chain reaction (rtRT-PCR). The rtRT-PCR assay has limitations in terms of cost and resources, especially in low-to-middle income countries.[2] Pool testing is an ideal strategy for screening purposes, contact tracing and population-based studies in such countries with low prevalence rate (< 10%).[3]

The concept of pooled testing was first developed by the economist Robert Dorfman in 1943 to detect syphilis in US soldiers during World War II.[4] Pooling strategies have also been widely used for the detection of hepatitis B and C viruses, human immunodeficiency virus (HIV) and *Neisseria gonorrhoeae*.[5] One of the major discussions of this method is to determine the pool size, as testing large numbers of pooled samples is not possible without an inherent loss of sensitivity. This study investigated the extent to which pooling was possible, while maintaining the detection capacity of weakly positive samples, during the laboratory diagnosis of COVID-19.

Methods

Study sample

This is an exploratory laboratory study. Nasopharyngeal and oropharyngeal swab samples previously collected at the Bandaranaike International Airport (BIA) from passengers for screening purpose were used for the study. Prior approval from the Molecular Diagnostic Laboratory (MDL), BIA was obtained to utilise tested and stored samples which had been forwarded from the laboratory from October 2020 to December 2020. Twenty (n = 20) samples with Ct values between 25 and 35 from known positives and 60 known negatives stored at -80 °C were used for the study.

Pooling of samples, RNA extracts and real time RT-PCR

The pooling was set at 2-, 4- and 8-fold dilution for both sample and RNA extracts. When pooling the samples, a 2-fold pool was prepared using 300 µL of a positive sample (n = 10) and 300 µL of a known negative sample; A 4-fold pool was prepared using 300 µL of the 2-fold pool and 300 µL of a new known negative sample; An 8-fold pool was prepared using 300 µL of the 4-fold pool and 300µL of a new known negative sample (Figure 1). Each pool was then subjected to RNA extraction using SpinStar™ Viral Nucleic Acid Kit 1.0 (ADT Biotech sdnbhd, Malaysia – Catalog No: 811803) according to the manufacturer's instructions.

For pooling of RNA extracts, the next set of 10 positive and 30 negative samples were subjected to RNA extraction. The viral RNA extracts were used to prepare the 2-, 4- and 8- fold pools of RNA extracts where, for the 2-fold pool, 20 µL of the known positive extract was pooled with 20 µL of known negative extract; For the 4-fold pool, 20 µL of the 2-fold extract was pooled with 20 µL of known negative extract, and for the 8-fold pool, 20 µL of the known 4-fold positive RNA extract was pooled with 20 µL of known negative extract. All neat and pooled samples and extracts were duplicated to assess reproducibility.

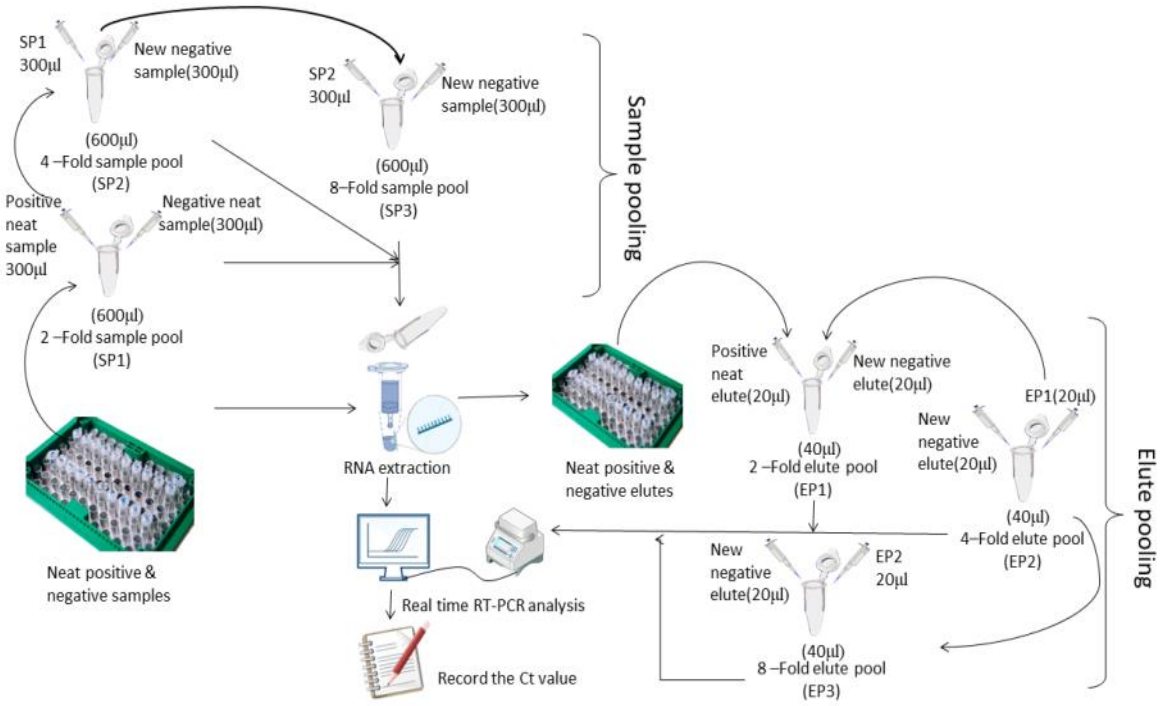

Figure 1. Experimental design for pooling of samples and RNA extracts

All pools and the neat samples derived from RNA extracts were tested by rtRT-PCR assay using Real Star® SARS-CoV-2 rtRT-PCR kit 1.0 (Altona Diagnostics GmbH, Germany) following the manufacturer's protocol using the CFX96™ cycler (BIORAD, Singapore); and their Ct values were recorded.

Statistical analysis

The detection capacity in sample and extract pools for SARS-CoV-2 RNA were determined by the rtRT-PCR. The Ct values of the rtRT-PCR for the pools were compared with that of the neat ones using paired sample *t*-test using MiniTab 19 and $P < 0.05$ was considered as statistically significant.

Results

Detection capacity for SARS-CoV-2 sample pooling

The overall detection of SARS-CoV-2 RNA in 2-, 4- and 8-fold sample pools were 100%, 90% and 50%, respectively when compared with the neat samples (Table 1). The detection capacity for SARS-CoV-2 RNA in the 2- and 4-fold sample pools was not significantly different, but it differed significantly for the 8-fold pool compared to the neat samples.

Comparison of Ct values between the neat and pooled samples / extracts are summarised in Table 2. SARS-CoV-2 RNA was detected in 2- and 4-fold pooled samples, even if the Ct value of a single sample was up to 35. However, in 8-fold pooled sample, the sensitivity decreased by 50%. In testing the 2-fold pooled samples, all were positive with minimal Ct variation for both E and S genes, while in 4-fold pooled samples, only one low positive sample (Ct = 35) was identified as negative. However, 8-fold pooled samples were tested , 5 positives were identified as negative in both replicas thus confirming the consistency of the results.

Table 1. Detection capacity for SARS-CoV-2 in sample and RNA extract pools.

Pools	Sample size	Test results		Sensitivity (95% CI)	False negativity
		Detected	Not detected		
Samples					
2-fold	10	10	0	100% ± 0	0
4-fold	10	9	1	90% ± 0.20	10%
8-fold	10	5	5	50% ± 0.44	50%
RNA extracts					
2-fold	10	10	0	100% ± 0	0
4-fold	10	10	0	100% ± 0	0
8-fold	10	10	0	100% ± 0	0

For these samples, the average of Ct value differences (Avg. ΔCt) between neat positive samples and pools were (Avg. ΔCt ± standard deviation) 1.69 ± 0.78 in pools of 2; 3.84 ± 1.47 in pools of 4 and 8.98 ± 2.24 in pools of 8. The difference in Ct values were calculated between neat and pooled samples, and it was noted that there was an increase in the ΔCt value with the increase in the number of samples in the pool. This increase was more pronounced in samples with low viral load in 8-fold pools (Table 2, Figure 2).

Table 2. Comparison of Ct values between neat and pooled samples.

Target Genes	Average Ct for neat	Average Ct value			Average ΔCt value (±*SD)		
		2-fold pools	4-fold pools	8-fold pools	Ct of 2-fold pool – *Ct_N	Ct of 4-fold pool – Ct_N	Ct of 8-fold pool – Ct_N
Samples							
S gene	30.98	32.67	34.82	39.96	1.69 ± 0.78	3.84 ± 1.47	8.98 ± 2.25
E gene	31.39	33.00	35.15	40.35	1.61 ± 0.83	3.76 ± 1.54	8.96 ± 217
RNA extracts							
S gene	27.19	28.20	29.37	30.24	1.01 ± 0.38	2.18 ± 0.82	3.05 ± 0.77
E gene	27.63	28.61	29.78	30.77	0.98 ± 0.34	2.15 ± 0.74	3.14 ± 0.74

*Ct_N – Ct value of the neat sample. *SD – standard deviation.

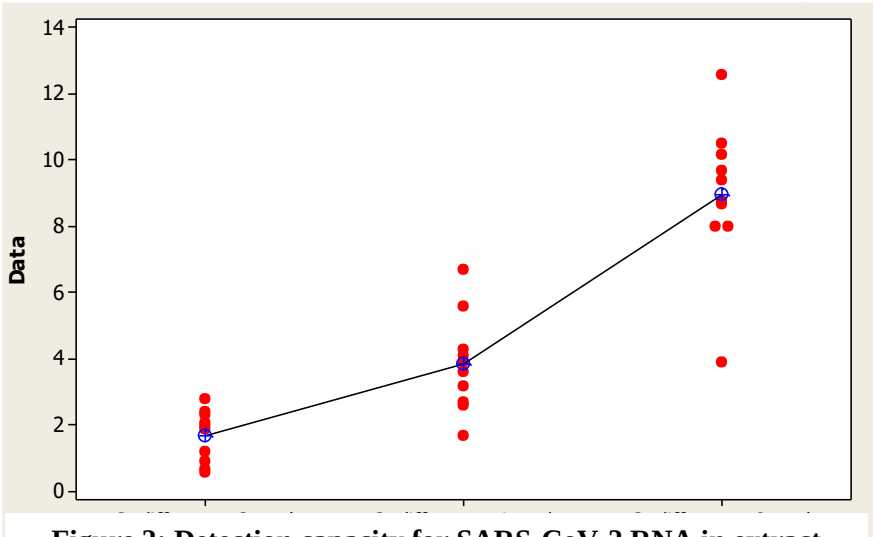

Figure 2: Detection capacity for SARS-CoV-2 RNA in extract pooling

Comparison of the mean Ct value of 2-, 4- and 8-fold pooled samples with their neat samples showed a significant difference ($P < 0.0001$). PCR inhibition was not observed in any of the nasopharyngeal swabs used in the present study, and minimal variations were observed in the Ct

values of all internal controls.

The overall sensitivity of pool testing with 2-, 4- and 8-fold pooled RNA extracts were 100% (Table 1) compared to neat RNA extracts. Comparison of the Ct values of the rtRT-PCR between the neat and pooled RNA extracts is summarised in Table 2. SARS-CoV-2 RNA was identified in positive extract pools of 2-, 4- and 8-fold dilution even if Ct values of the single extract was up to 35. Two, 4- and 8-fold extract pools were rtRT-PCR positive for SARS-CoV-2 RNA with minimum Ct variations.

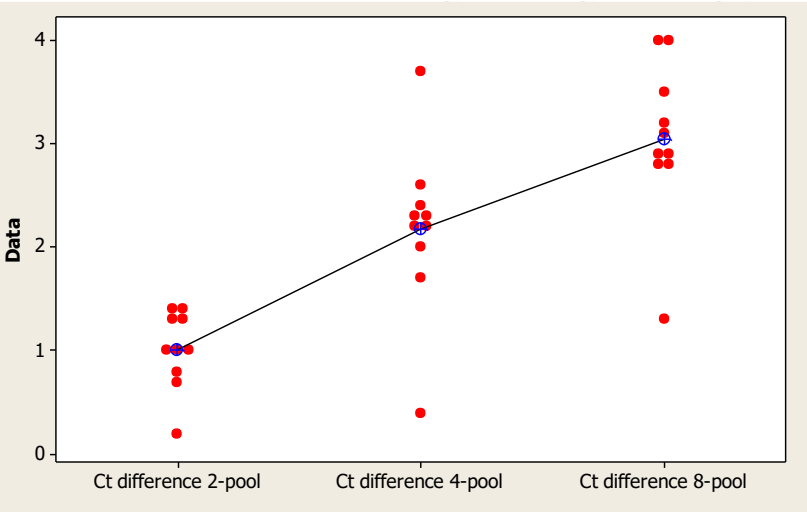

Figure 3. Individual Value Plot showing the increase in Ct value with RNA extract pooling.

For these RNA extracts, the Avg. ΔCt between positive individual extracts and the pooled extracts were 1.01 ± 0.38 in pools of 2, 2.18 ± 0.82 in pools of 4 and 3.05 ± 0.77 in pools of 8. The ΔCt was calculated between individual and pooled RNA extracts. The current study showed an increase in the ΔCt value with the increase in the number of samples included in the pool (Table 2, Figure 3). This difference was almost equal for all pooled RNA extracts, and this reduced the likelihood of false negativity. Minimal variations were observed in all the internal control Ct values in the rtRT-PCR of the RNA extract pools. The statistical comparison of the mean Ct value of 2-, 4- and 8-fold pooled extracts with their neat extracts also showed a significant difference between pooled and neat extracts ($P < 0.0001$), however, there was no difference in the detection ability.

Discussion

Pooling of nasopharyngeal swabs and their RNA extracts for SARS-CoV-2 rtRT-PCR testing has been a promising strategy for screening a large number of samples.[6] Different studies use different techniques of pooling and pool sizes.[5,7–9] This study shows that pooling of nasopharyngeal samples before extraction in 2- or 4-fold is more reliable than in 8-fold pools. It was also demonstrated that pooling of extracted RNA in 2-, 4- and 8-fold could be achieved without compromising the sensitivity. In this study, pooling of samples before extraction showed a decrease in the detection of SARS-CoV-2 RNA when the pool size was increased from 2-fold to 8-fold. Two-fold sample pooling had a sensitivity of 100% compared to the individual samples tested by rtRT-PCR. A sensitivity of 90% was observed in 4-fold sample pooling with a 10% false negativity. A drop in 50% sensitivity was observed for 8-fold sample pooling with 50% false negativity. The average Ct value of the neat samples was higher than that of the neat RNA extracts in this study and it could be a reason for the significant drop in the sensitivity in sample pooling when the pool size was increased. Pooling of samples have been suggested to help manage workload and to enhance the testing capacity. However, application of this strategy in clinical diagnosis has the potential to increase the rate of false negativity, especially when pooling low positive samples.[10]

Several studies have reported similar results and some studies claim pooling to be an effective strategy.[3,11–15] The viral load is an important factor affecting the detection in pooled testing. Furthermore, the test sensitivity of rtRT-PCR of pools may differ with the sensitivity of the rtRT-PCR assay used, techniques of sample collection, dilution used, type of sample and temperature at which the sample is transported / stored.[11,15] In this study, it was noted that the probability of detection of the positive pool decreased as the Ct value of the single positive sample in the pool increased (lower viral load). On average, the Ct values obtained with the 2-fold sample pool testing exceeded the individual sample testing by 1.69 ± 0.78 cycles while Ct values obtained with the 4-fold sample pools exceeded the individual sample testing by 3.84 ± 1.47 cycles and Ct values obtained with the 8-fold sample pools exceeded the individual sample by 8.98 ± 2.24 cycles. Comparison of means of Ct values between neat samples and their pools produced significant *P*-values (< 0.0001). However, the change in Ct values was 3.84 ± 1.47 for 4-fold pool and thus, a shift in the cut off by +3 did not significantly compromise the detection capacity. High Ct values in sample pooling are due to the low virus load in pooled samples. Moreover, high Ct value may be due to improper sample collection, or sampling from a person in early or late stages of infection when the viral load is low. These samples may miss detection when they are pooled and this in turn may pose a threat to infection control efforts during an evolving pandemic, hence, the question remains unanswered - whether or not it is safe to miss such cases while combating an infection.

Data from this study also demonstrated pooling of extracted RNA can be increased up to 8-fold without compromising sensitivity. However, the labour-intensive RNA extraction of each sample would lead to delays in detection and decision making apart from using additional resources, thereby adding to the cost. Nevertheless, RNA pooling will be helpful in attaining a reduced test turn-around time by avoiding the need to perform additional extractions if positive pools are detected, enabling testing of the individual extracted RNA without delay. In RNA extract pooling, the Ct values obtained with the 2-fold pool exceeded the individual testing by 1.01 ± 0.38 cycles, while Ct values obtained with the 4-fold pool exceeded the individual extract testing by 2.18 ± 0.82 cycles and Ct values obtained with the 8-fold pool exceeded the individual extract testing by 3.05 ± 0.77 cycles. The difference between means of Ct values of pooled RNA extracts and neat RNA extracts was also statistically significant ($P < 0.0001$). However, the detection capacity remained the same for all three RNA extract pools compared to the neat samples. In agreement with the current study findings, Gupta *et al*.,[16] reported that pool testing with RNA extracts can easily detect even up to a neat positive sample with Ct value as high as 38 in a pool of 8. This suggests that as many as 8-fold pools of RNA extracts can be pooled without compromising the capacity of detection and thus appears to be more suitable for mass screening when a necessity arises.

Based on these results, either 4-fold pooling of samples or 8-fold pooling of RNA extracts increases the testing capacity and detects positive samples with at least 90% accuracy. In contrast to developed countries, detailed published data on pool testing in resource-limited settings are lacking. Laboratories must adjust the size of the pool according to the prevalence of infection in each setting.[17] Conversely, pooling is not effective during periods of high prevalence (>15%) as every pool will yield a positive result and thus demand subsequent separate re-testing of every sample in the pool

Limitations of the current study include conducting the study in a single laboratory and evaluating just 3 different pool sizes and 20 positive samples. This was due to constraints in

resources and well-resourced studies in the are needed to validate sample pooling and their cost-effective contributions for the detection of different viruses.

Conclusion

Large scale screening of asymptomatic individuals for SARS-CoV-2 can be maximised with optimal use of resources by 2- or 4-fold pooling of samples or 4- or 8-fold pooling of RNA extracts without significantly compromising the detection sensitivity.

Declaration

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Authors' contributions:

MIH Sulthan:	Laboratory analysis, data collection and formal analysis, writing-original draft.
SRM Shihab:	Statistical analysis, data collection and formal analysis, writing-original draft.
BN Iqbal:	Data collection and formal analysis, writing-original draft.
AP Pitawala:	Project supervision and resources.
F Noordeen:	Conceptualization, supervision, critical review and editing of the manuscript.

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