Section: Molecular Biology

Original Research Article

IMPACT OF SPECIMEN POOLING ON CYCLE THRESHOLD VALUES IN SARS-COV-2 RT-PCR TESTING

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ABSTRACT

Background: The concept of pooled testing is based on the Dorfman theory of specimen pooling. The performance characteristics of the RT PCR kits are generally estimated by testing unpooled specimen, and these parameters could be impacted when testing pooled clinical specimens. **Aims and Objectives**: The study was conducted at a tertiary care center to estimate the change in Ct values (ΔCt) of positive specimens when tested using pooling strategy of varying pool sizes, i.e. five, 10, 15 and twenty. **Materials and Methods:** The present study was a cross sectional study in which known positive specimen were mixed with confirmed negative specimen in pools of five, 10, 15 and 20 for Sars-CoV-2 RT-PCR testing. **Results:** The specimens were tested with three kits and the ΔCt for the pool of five, 10, 15 and 20 was 0.5, 0.7 (pool of five), 2.6, 2.7 (pool of 10), 5.5, 5.6 (pool of 15) and 11.3, 11.9 (pool of 20) for ORF1ab and N gene using the Meril COVID-19 one step RT- PCR kit. Similar results were obtained using two more kits (COVID-19 RT-PCR Kit by Q-line Molecular and DiAGSure^{TM} nCoV-19 kit). **Conclusions**: The ΔCt of approximately three for a pool size of 10 suggests that low viral load specimens could be missed by increasing the pool size beyond five. In the event of resource constraints, suitable pooling size of upto five should be employed for RT-PCR testing so that infected patients with low viral counts are not missed.

INTRODUCTION

The coronavirus disease-2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) started in December 2019 from China.[1] World Health Organization (WHO) declared it a pandemic on March 11, 2020 and an urgent need to scale up the rate of testing was realized in most countries around the globe.[2] Amongst the available testing modalities, conventional RT- PCR was the preferred and most widely used test for COVID-19 diagnosis.[3,4] RT-PCR is an expensive test and requires a well-equipped molecular laboratory with trained manpower. Many countries experienced acute shortages of diagnostic kits, molecular laboratories and trained staff. The manufacturers of molecular testing kits and consumables also struggled to keep with the demand.

Realizing the need for the scale of testing in India, the concept of pooled specimen testing was put in place in by ICMR. This was one of the several other experimental modalities that were tried in anticipation of finding innovative methods of tackling the pandemic such as new vaccines, plasma therapy etc.[5,6] The concept of pooled testing is based on the Dorfman theory,[7] of specimen pooling in the era of molecular testing.[8,9] In a study conducted by Abdalhamid et al,[10] the authors used a web- based application and determined the most efficient pool size to be five specimens when the incidence rate of SARS-CoV-2 infection is 10% or less and concluded that group testing will result in saving of reagents and increase in the testing capability of at least 69%. Several countries were performing pooled specimen testing for COVID-19; however, the impact of specimen pooling on the cycle threshold (Ct) value was not widely available at that time. Ct is defined as "cycle number where the fluorescent signal from DNA amplification crosses a set detection threshold" which is a measure of the viral load of the specimen. The COVID-19 RT PCR kit manufacturers estimate the performance characteristics of the kits by testing unpooled specimen, and these parameters could be impacted when testing pooled clinical specimens. Therefore, the present study was conducted at a tertiary care center to estimate the change in Ct values (ΔCt) of positive specimens when tested using pooling strategy of varying pool sizes, i.e. five, 10, 15 and twenty. The secondary objective of the study

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was to compare the performance characteristics of the available kits from various manufacturers when performing during pooled specimen testing.

MATERIALS AND METHODS

The present study was a prospective study was carried out between November 2021 and March 2022 in the Molecular Laboratory of a tertiary care centre from Western India. The laboratory is recognized by the Indian Council of Medical Research (ICMR) for SARS-CoV-2 RT-PCR testing and holds accreditation from the National Accreditation Board for Testing and Calibration Laboratories (NABL). A total of 94 nasopharyngeal swab specimens preserved in viral transport medium (VTM) were included, comprising of 47 previously confirmed COVID-19 positive and 47 confirmed negative cases. All specimens were retrieved from the biorepository of the ICMR-approved molecular laboratory, where they had been stored at –80°C prior to analysis.

Rechecking the performance of RT-PCR test on stored (-80°C) known clinical specimen

Nasopharyngeal swab specimens preserved in VTM were thawed at room temperature and subsequently handled within a Class II biosafety cabinet in the designated specimen processing room immediately after removal from the –80°C deep freezer. Each VTM specimen was then subjected to viral nucleic acid extraction using the Hi-Media RNA extraction kit. The extracted RNA was amplified using Ag Path One-Step RT-PCR reagents (ThermoFisher Scientific) along with primer and probe sets targeting the E gene, RdRP gene, and RNase P as per World Health Organization (WHO) protocol.[11] This procedure was performed to evaluate the integrity and performance of stored clinical specimens prior to their inclusion in the study. The negative specimens were included in the study after routine testing on the same day so no retesting was required for such specimens.

Specimen Inclusion and Exclusion Criteria

Positive specimens with cycle threshold (Ct) values between 18 and 25 and negative specimens without evidence of contamination upon re-evaluation were included. Positive specimens with a cycle threshold (Ct) value of <18 or >25, as well as contaminated negative specimens, were excluded from the study.

Pool preparation of clinical specimens

A total of 47 pooled specimens were prepared with pool sizes of five, 10, 15, and 20. For the pool size of five, each pool was constituted by combining 60µl of viral transport medium (VTM) from one RT-PCR–confirmed SARS-CoV-2 positive specimen with 60µl VTM from each of four negative specimens in a sterile Eppendorf tube, yielding a final volume of 300µl. Similarly, pools of size 10 were prepared by mixing 30µl VTM from one positive specimen with 30µl VTM from nine negative specimens. Pools of size 15 were created using 20µl aliquots (one positive and 14 negative specimens), while pools of size 20 were prepared using 15µl aliquots from one positive and 19 negative specimens, ensuring that the final volume of each pooled specimen was consistently maintained at 300µl. The pooled 300µl VTM served as the starting material for nucleic acid extraction.

Selection of RT-PCR kits

A survey was undertaken to evaluate commercially available RT-PCR kits with respect to their regulatory approvals (US-FDA, CE, and ICMR, India), analytical sensitivity (lower limit of detection), practical usage, availability within India, and compatibility across different PCR platforms. The following kits were included in the present study. Table 1]

Table 1: RT –PCR kits consider for the evaluation

S. No.	Kit name	Manufacturer detail	Gene target	Ct cut off
1	Meril COVID-19 one step RT- PCR kit	Meril Diagnostic	ORF1ab, N gene	Ct< 40 Positive
2	Coronavirus (COVID-19) RT- PCR Kit	Q-line Molecular	ORF1ab, N gene	Ct< 35 Positive
3	DiAGSure™ nCoV-19 detection Assay	GCC Biotech	ORF1ab, E gene	Ct< 40 Positive

Nucleic acid extraction and RT PCR

RNA extraction was performed for each pooled specimen using the Hi-Media Viral RNA Extraction Kit. The eluted RNA was subsequently processed for RT-PCR analysis using the master mix reagents mentioned in Table 1. All RT-PCR tests were conducted on the Applied Biosystems QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific). Thermocycling conditions and interpretation of results were carried out strictly according to the manufacturer's instructions.

Statistical Analysis: The data was entered in Microsoft Excel and SPSS (IBM Corp., USA) was used for statistical analysis. Friedman's chi-square test was used to assess the significance of the change in Ct values whereas the impact of the increase in Ct value was assessed by the Wilcoxon signed rank test.

Ethical Clearance

The study was conducted after obtaining due ethical approval from the Institutional Ethics Committee vide Approval No. 045/ZMCH/3rd IEC/02/12/21; Registration No. ECR/1464/Ins/GJ/2020.

RESULTS

The mean Ct value of unpooled positive specimens were 19.78 for ORF1ab gene and 20.5 for N gene. The specimen were tested with the Meril COVID-19

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one step RT- PCR kit and the mean Ct value for the pools of five was 20.5 and 21.2 (ORF1ab and N gene), 22.6 and 23.2 (ORF1ab and N gene) for the pool of 10, 25.5 and 26.1 (ORF1ab and N gene) for the pool of 15 and 31.3 and 32.4 (ORF1ab and N gene) for the pool of twenty. The ΔCt was statistically significant (P <0.001). However, the results were interpreted as positive for all the pools. The results are shown in Fig 1A. The comparison of each step up of pool size is shown in Fig 1B and the difference was statistically significant (P<0.01). The ΔCt upon dilution in a pool of five, 10, 15 and 20 was 0.5, 0.7 (pool of five), 2.6, 2.7 (pool of 10), 5.5, 5.6 (pool of 15) and 11.3, 11.9 (pool of 20) for ORF1ab and N gene.

The specimen were then tested with the Coronavirus (COVID-19) RT- PCR Kit (Q-line Molecular) and the mean Ct value for the pools of five was 21.3 and 21.4 (ORF1ab and N gene), 24.8 and 23.5 (ORF1ab and N gene) for the pool of 10, 26.9 and 27.6 (ORF1ab and N gene) for the pool of 15 and 32.7 and 33 (ORF1ab and N gene) for the pool of twenty. The ΔCt was statistically significant (P <0.001). However, the results were interpreted as positive for all the pools. The results are shown in Fig 1E. The comparison of each step up of pool size is shown in Fig 1F and the difference was statistically significant (P<0.01). The ΔCt upon dilution in a pool of five, 10, 15 and 20 was 1.3, 0.9 (pool of five), 4.8, 3.0 (pool of 10), 6.9, 7.1 (pool of 15) and 12.7, 12.5 (pool of 20) for ORF1ab and N gene.

The specimen were finally tested with the DiAGSureTM nCoV-19 detection Assay (GCC Biotech) and the mean Ct value for the pools of five was 20.6 and 21.3 (ORF1ab and N gene), 22.6 and 23.2 (ORF1ab and N gene) for the pool of 10, 25.4 and 26 (ORF1ab and N gene) for the pool of 15 and 31.1 and 32.2 (ORF1ab and E gene) for the pool of twenty. The ΔCt was statistically significant (P <0.001). However, the results were interpreted as positive for all the pools. The results are shown in Fig 1C. The comparison of each step up of pool size is shown in Fig 1D and the difference was statistically significant (P<0.01). The ΔCt upon dilution in a pool of five, 10, 15 and 20 was 0.6, 0.8 (pool of five), 2.6, 2.7 (pool of 10), 5.4, 5.5 (pool of 15) and 11.1, 11.7 (pool of 20) for ORF1ab and N gene. The performance characteristics of the three kits were compared and the three kits performed well with similar levels of sensitivity and specificity. All three kits detected 100% positive pools giving a sensitivity and specificity values of 100% respectively.

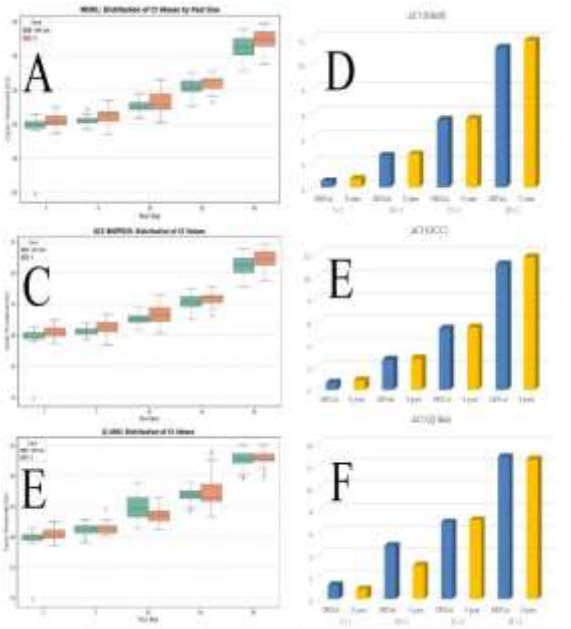

Figure 1: The distribution of Ct values (A, C and E) and the change in Ct (ΔCt) upon pooling of varying sizes (B, D, F)

DISCUSSION

In the present study, we evaluated the effect of specimen pooling on positivity rates using three diagnostic kits from different manufacturers. Our findings indicate that specimens remained positive even when pooled up to a size of twenty. However, as expected, the Ct values increased following dilution of positive specimens, reflecting reduced viral concentration. As per the standard formula for estimation of change in Ct value upon dilution i.e. $\Delta Ct = \log2(n)$,[12] where n represents the pool size, the expected change in Ct value for a pool size of five, 10, 15 and 20 were 2.3, 3.3, 3.9 and 4.3 respectively. The ΔCt in the present study, however, was not in accordance with this formula and the pools of five yielded a much smaller change whereas the pools of 15 or more yielded a much bigger change.

Interestingly, studies have estimated the impact of pooling on Ct value during the COVID-19 pandemic phase. Barak et al,[13] reported the findings from a study where eight and five specimen pools were practiced during the COVID-19 period depending upon the positivity rate. They estimated that the eight specimen pool had a shift in Ct value by approximately 2.9 which is close to the estimate of three cycle loss using the mathematical formula discussed above. Another study by Agoti et al demonstrated that the shift in Ct value was around 1.59 but the pooled strategy doubled the testing capacity and halved the kit/ reagent consumption.[14] The impact of pooling could reflect fairly or acceptable but could change the interpretation of a specimen entirely if the viral load is on the lower side. A ΔCt of three or more could lead to a false negative result for pooled testing containing low viral load specimen. For reference, the Meril COVID-19 one

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step RT- PCR kit has a Ct cut off of 40 for labelling a specimen as positive. Theoretically, a ΔCt of three or more could change the interpretation of a positive specimen with a low viral load with a Ct of thirty eight.[15] A study by Abdelrazik et al,[15] included varying pools of four, eight and 10 in which the authors reported a ΔCt of three for a pool size of 10 but the pools consisting of a low viral load specimen were missed and falsely labelled as negative. Importantly, the rate of false negative in these pools were as high as 9% in contrast to zero in a pool of four and 1% for a pool of eight. Similar findings were reported by Mahmoud et al,[16] Perchetti et al,[17] and Allicock et al.[18] In the present study,

An important limitation of the study is the non-inclusion of low viral load, confirmed positive specimens (Ct value between 30-39). Such specimens may potentially yield false-negative results upon dilution which might change the interpretation as the pool size increased. The non-inclusion of low viral load specimens was due to the inability of such specimen to allow the estimation of ΔCt as per the approved manufacturer instructions provided with the RT PCR testing kits. Another reason for their non-inclusion was due to the higher chances for the need of retesting such specimens to address such requests from the health authorities. The prevailing situation warranted the requirements for storing positive specimens for a minimum defined period and the diagnostic laboratory dictated a longer storage times for low positive specimens.

CONCLUSION

The present study estimates that the pooling strategy is effective for a pool size of upto 20 for specimens having a Ct value between 18-25 but the ΔCt of approximately three for a pool size of 10 suggests that low viral load specimens could be missed by increasing the pool size beyond five. In the event of resource constraints during a pandemic, suitable pooling size of upto five should be employed for RT-PCR testing so that infected patients with low viral counts are not missed.

REFERENCES

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. N Engl J Med. 2020;382:727-733.
2. Ganesan C, Vandavasi M, Dhaigude AS. Accelerating COVID-19 testing: An experimental approach. J Public Aff. 2022:e2827. doi: 10.1002/pa.2827.
3. Tang YW, Schmitz JE, Persing DH, Stratton CW. Laboratory diagnosis of COVID-19: current issues and challenges. J Clin Microbiol. 2020;58:e00512.
4. Dramé M, Tabue Teguo M, Proye E, Hequet F, Hentzien M, Kanagaratnam L, et al. Should RT-PCR be considered a gold standard in the diagnosis of Covid-19? J Med Virol. 2020;92: 2312-2313.
5. Bansal Y, Chand P, Bansal N, Singh P. Vaccine hesitancy after taking first dose of COVID-19 vaccine: A challenge for COVID-19 vaccination programme in India. J Family Med Prim Care. 2022;11:2201-2206.
6. Bansal N, Raturi M, Bansal Y. Covid-19 convalescent plasma therapy: Analyzing the factors that led to its failure in India. Transfus Clin Biol. 2021;28:296-298. doi: 10.1016/j.tracli.2021.05.009.
7. Dorfman R. The detection of defective members of large populations. Annal Math Stat. 1943;14:436-440.
8. Torres I, Albert E, Navarro D. Pooling of nasopharyngeal swab specimens for SARS-CoV-2 detection by RT-PCR. J Med Virol. 2020;92:2306-2307.
9. Garg J, Singh V, Pandey P, Verma A, Sen M, Das A, et al. Evaluation of sample pooling for diagnosis of COVID-19 by real time-PCR: A resource-saving combat strategy. J Med Virol. 2021;93:1526-1531.
10. Abdalhamid B, Bilder CR, McCutchen EL, Hinrichs SH, Koepsell SA, Iwen PC. Assessment of Specimen Pooling to Conserve SARS CoV-2 Testing Resources. Am J Clin Pathol. 2020;153:715-718.
11. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020;25:2000045. doi: 10.2807/1560-7917.ES.2020.25.3.2000045.
12. Millioni R, Mortarino C. Test Groups, Not Individuals: A Review of the Pooling Approaches for SARS-CoV-2 Diagnosis. Diagnostics (Basel). 2021;11:68. doi: 10.3390/diagnostics11010068.
13. Barak N, Ben-Ami R, Sido T, Perri A, Shtoyer A, Rivkin M, et al. Lessons from applied large-scale pooling of 133,816 SARS-CoV-2 RT-PCR tests. Sci Transl Med. 2021;13(589):eabf2823. doi: 10.1126/scitranslmed.abf2823.
14. Agoti CN, Mutunga M, Lambisia AW, Kimani D, Cheruiyot R, Kiyuka P, et al. Pooled testing conserves SARS-CoV-2 laboratory resources and improves test turn-around time: experience on the Kenyan Coast. Wellcome Open Res. 2021;5:186. doi: 10.12688/wellcomeopenres.16113.2.
15. Abdelrazik AM, Said MNE, Abdelaziz HM. Evaluation of pooling strategy of SARS-CoV-2 RT-PCR in limited resources setting in Egypt at low prevalence. Comp Clin Path. 2023;32:375-381. doi: 10.1007/s00580-023-03445-6.
16. Mahmoud SA, Ibrahim E, Thakre B, Teddy JG, Raheja P, Ganesan S, et al. Evaluation of pooling of samples for testing SARS-CoV- 2 for mass screening of COVID-19. BMC Infect Dis. 2021;21:360. doi: 10.1186/s12879-021-06061-3.
17. Perchetti GA, Sullivan KW, Pepper G, Huang ML, Breit N, Mathias P, et al. Pooling of SARS-CoV-2 samples to increase molecular testing throughput. J Clin Virol. 2020;131:104570. doi: 10.1016/j.jcv.2020.104570.
18. Allicock OM, Yolda-Carr D, Todd JA, Wyllie AL. Pooled RNA-extraction-free testing of saliva for the detection of SARS-CoV-2. Sci Rep. 2023;13:7426. doi: 10.1038/s41598-023-34662-2.

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