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Original Research Article

Evaluation of the Dilution Effect of Respiratory Specimen Pooling on the Sensitivity of SARS-COV 2 RT-PCR Tests

Priyanka Saha[1], Kuhu Pal[2], Srijita Ghosh[3], Retina Paul[4], Satakshi Basu [5], Arpita Sinha[6], Joydeep Karmakar[7]

[1]Assistant Professor, Department of Microbiology, College of Medicine and JNM Hospital, Kalyani, West Bengal

[2]Professor and HOD, Department of Microbiology, College of Medicine and JNM Hospital, Kalyani, West Bengal

[3]Demonstrator, Department of Microbiology, Medical College, Kolkata

[4]Associate Professor, Department of Microbiology, College of Medicine and JNM Hospital, Kalyani, West Bengal

[5]Molecular Biologist, Department of Microbiology, College of Medicine and JNM Hospital, Kalyani, West Bengal

[6]Demonstrator, Department of Microbiology, College of Medicine and JNM Hospital, Kalyani, West Bengal

[7]Molecular Biologist, Department of Microbiology, College of Medicine and JNM Hospital, Kalyani, West Bengal

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Corresponding Author: Dr. Retina Paul
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Abstract

Introduction: The Covid-19 pandemic affects worldwide health and causes economic disasters. Testing sample in pool is the best strategy for surveillance. Pooling of samples reduces cost and turn-around time of RT-PCR test if done in the setting of low prevalence. But at the same time, it may give false negative results due to dilution effect, especially in higher range of cycle threshold value (Ct value). So, it is recommended that every RT-PCR laboratory should optimize pool size for improving test output utilizing minimum resources and without compromising sensitivity of the test.

Aim: Current study was conducted to evaluate the dilution effect of pooling of samples on the sensitivity of RT-PCR Test and compare Ct value of positive pool with that of individual positive sample.

Materials and Methods: Wide range of positive samples starting from highest Cycle threshold value(Ct) 35 to lowest 15(i.e. 21 sets different Ct value positive specimen) were tested for 7 different sets of pool such as 3,5,10,15,20,25,30 specimen pool for each gene(N and ORF1 ab) separately. After pool formation RNA extraction was done by both manual and automated extraction method followed by detection of COVID-19 target gene by RT-qPCR.

Results: All pooled specimens of both genes were tested positive until and below Ct value 24 by both extraction methods. Dilution effect of pooling were noticed from Ct value 25 upwards. Sensitivity of pooled specimen decreases with increasing pool size. 100% sensitivity observed up to 10 pool and 15 pool sample in manual and automated extraction method respectively.

Conclusion: Pool testing is the best strategy to carry out population-wide surveillance as new variant of COVID-19 is coming up frequently. Our study indicates we can increase pool size upto15 specimen pool with minimal loss of sensitivity. Current study also observed sensitivity of automated extractor outweighs manual extraction method. COVID-19 no longer constitutes a public health emergency of international concern but being a highly contagious disease, it has the potential to cause rapid outbreak especially in healthcare settings. So periodic surveillance of every ward and every new patient on admission should be incorporated in Hospital infection control programme of every Health care setting.

Keywords: Pool testing, SARS-CoV-2, RT-PCR test.

Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) originated from Wuhan, China [1] and has affected all the country's citizens worldwide since December 2019 [2, 3]. World Health Organisation (WHO) declared COVID-19 as a pandemic during March 2020, sending countries across the world into shutdowns to stop the spread. Although, during last year WHO declared an end to global pandemic due to COVID-19, new cases and deaths are still being recorded across the world. As of 31st March, 2025, there have been 777,950,273 confirmed cases of COVID-19, including7,096,650 death and within last 28 days cases 206,279 including 1,019 deaths have been reported to WHO [4]. The Covid-19 pandemic affects worldwide health and causes economic disasters [5].

Real-time reverse transcription-polymerase chain reaction (RT-PCR)-based molecular assays for various SARS-CoV-2 gene targets are the mainstay of diagnosis for COVID-19 at present [6,]. Real time RT-PCR assay is a 2 steps diagnostic procedure. RNA extraction from nasopharyngeal and oropharyngeal swab sample is the first step, followed by amplification of target genes(E/N/RdRp/ORF) and real time detection by fluorophores. So, Lot of resources in terms of manpower and reagents are required to complete this assay. Moreover, it takes almost a whole day from specimen collection to delivery of results. Thus, many countries discontinue COVID-19 testing due to resource constrain. But according to WHO, COVID-19 remains a major threat, and it suggests to all member countries not to dismantle their established COVID-19 infrastructure. It is crucial to sustain early warning, surveillance and reporting, variant tracking, early clinical care provision, administration of vaccine to high-risk groups, improvements in ventilation, and regular communication[7, 8].

Testing sample in pool is the best strategy for surveillance. Indian council of medical research had also recommended pool testing of 3-5 samples in an area with low positivity rate (<2%) for the purpose of surveillance. According to this advisory[9],all individual samples in a negative pool to be regarded as negative. Deconvoluted testing is recommended if any of the pool is positive. Till now pooling of more than 5 samples is not recommended to avoid the effect of dilution leading to false negatives.

Pooling of samples reduces cost and turnaround time of RT-PCR test if done in the setting of low prevalence. But at the same time, it may give false negative results due to dilution effect, especially in higher range of cycle threshold value (Ct value). So, it is recommended that every RT-PCR laboratory should optimize pool size for improving test output utilizing minimum resources and without compromising sensitivity of the test. Our study aimed to evaluate the dilution effect of pooling of samples on the sensitivity of RT-PCR Test and to determine an optimal pool size maintaining sensitivity

Materials and Methods

This cross-sectional observational study was done from July2022- December 2022 after taking clearance from ethics committee at RT-PCR laboratory. Our study included previously tested known positive (2 target genes detected according to manufacturer's instruction) and negative for SARS-CoV-2 nasopharyngeal swabs in viral transport media (VTM) which were stored at -80oC. Laboratory confirmed inconclusive samples(1 target gene is positive) and those having inadequate volume were excluded from this study.

Specimen pooling

The present study worked with 7 different sets of pool such as 3,5,10,15,20,25,30 specimen pool for each gene(N and ORF1 ab) separately. Wide range of positive samples starting from highest Cycle threshold value (Ct) 35 to lowest 15(i.e 21 sets different Ct value positive specimen) were tested for every pool size. Each of the 21 sets(3 positive specimen of individual gene at each Ct value) positive specimen was mixed sequentially with the negative sample of seven dilutions series with ratio of 1:2, 1:4, 1:9, 1:14, 1:19, 1:24, 1:29 to form a 3,5,10,15,20,25,30 specimen pool respectively. A 66 µl of each positive sample was mixed with the 134 µl of negative samples to make 3 sample pool. Likewise,40ul, 20 µl, 14 µl, 10 µl, 8 µl, 6µl, of each positive samples were added to 160ul, 180 µl, 186 µl, 190 µl, 192 µl, and 194 µl negative samples to prepare5,10,15,20,25,30 sample pool respectively for a final volume of 200ul.(0.5 µl measurement was rounded up to facilitate the experiment with more accuracy). Pool size couldn't be increased > 30 specimen pooling according to Regenet. al. [10].

RNA extraction

200 µl of the sample was further processed for viral nucleic acid extraction by manual column-based extraction(X-Spin Viral RNA Extraction Kit by AXIBIO) and Automated RNA extraction was performed with Hipura® Viral RNA Automated Extraction Combi Kit (HIMEDIA) as per the manufacturer's protocol. Detection of target covid -19 RNA with qRT-PCR.

The 10 µl of the extracted RNA elute was subjected to RT-qPCR for the qualitative detection of SARS-CoV-2 RNA employing CoviPath™ COVID-19 RT-PCR Kit (Thermo Fisher Scientific) utilising CFX96™ Real-Time PCR System and C1000™ Thermal Cycler (BIO-RAD). Reactions were set sequentially at 25°C for 2 min for UNG (Uracyl N-Glycosylase) incubation,53°C for 10 min for reverse transcription, activation period of 2 min in 95°C, denatured in 95°C for 3 seconds and then 40 cycles of annealing were carried out for 60°C for

30 s using fluorescein amidite parameter for two genes. This assay targets the detection of the N gene and ORF1ab gene[11]. Data analysis-Data was tabulated and analysed in Microsoft Excel sheet. Sensitivity was calculated by Med Calc free trial version (https://www.medcalc.org/calc/diagnostic_test.php)

Results

We have tested separate 7 sets of pool for both N and ORF 1ab gene starting from highest Ct to lower values. Note that a low Ct value indicated the presence of higher amounts of viral RNA and high Ct values indicated lower amounts[12]. Pools were considered positive if tested positive for any one of the two target genes(N and ORF1ab).

To evaluate the effect of dilution, Ct value of original positive specimen was subtracted from the Ct value of different pooling dilution and mean difference in Ct value were calculated(Table 1and Table 2).

Table 1: Mean Ct difference of target N gene at each dilution in manual and automated extraction method

Pool size	Manual extraction		Automated extraction	
	Mean Ct difference	Range	Mean Ct difference	Range
3P	1.70	1.02 to 2.66	1.27	-0.58 to 2.72
5P	3.22	1.72 to 4.55	2.50	0.86 to 4.95
10P	3.93	2.99 to 6.51	3.71	1.74 to 7.03
15P	6.00	4.49 to 7.64	4.74	2.6 to 7.44
20P	7.13	4.62 to 8.85	6.38	3.73 to 8.72
25P	8.46	6.66 to 11.11	8.04	4.85 to 11
30P	9.31	7.42 to 12.19	9.67	7.03 to 13.08

Table 2: Mean Ct difference of target ORF1ab gene at each dilution in manual and automated extraction method

Pool size	Manual extraction		Automated extraction	
	Mean Ct difference	Range	Mean Ct difference	Range
3P	1.32	-0.38 to 2.41	1.39	-0.62 to 3.04
5P	2.89	1.26 to 4.51	2.47	0.59 to 4.44
10P	4.83	2.82 to 6.35	3.81	1.52 to 7.15
15P	6.71	4.48 to 8.52	5.33	3.53 to 9.1
20P	8.64	6.28 to 10.78	6.98	4.9 to 10.64
25P	10.59	8.73 to 12.57	8.68	6 to 12.48
30P	11.71	9.94 to 14.32	10.41	7.43 to 14.2

Table: 3. Comparison of RNA Detection Efficiency across Different Pool Sizes and Ct Values Using Manual and Automated RNA Isolation Methods

RNA Isolation Method	Pool size	Ct values											
		24 (N = 6)	25 (N = 6)	26 (N = 6)	27 (N = 6)	28 (N = 6)	29 (N = 6)	30 (N = 6)	31 (N = 6)	32 (N = 6)	33 (N = 6)	34 (N = 6)	35 (N = 6)
Manual	3P	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	5P	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	10P	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	66.66%
	15P	100%	100%	100%	100%	100%	100%	100%	100%	100%	66.66%	0%	0%
	20P	100%	100%	100%	100%	100%	100%	100%	100%	50%	0%	0%	0%
	25P	100%	100%	83.33%	66.66%	50%	50%	0%	0%	0%	0%	0%	0%
	30P	100%	66.66%	50%	16.67%	0%	0%	0%	0%	0%	0%	0%	0%
Automated	3P	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	5P	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	10P	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	15P	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	66.66%
	20P	100%	100%	100%	100%	100%	100%	100%	100%	100%	83.33%	33.33%	0%
	25P	100%	100%	100%	100%	100%	100%	100%	100%	100%	33.33%	0%	0%
	30P	100%	100%	100%	100%	66.66%	50%	50%	33.33%	0%	0%	0%	0%

All pooled specimens of both genes were tested positive until and below Ct value 24 by both extraction methods. Dilution effect of pooling were noticed from Ct value 25 upwards. Percentage of positive specimen at each Ct value (≥24) were calculated combining the results of both target genes in different pooling dilutions (Table 3).

Table 3: Percentage of positive specimens for each Ct value(starting from Ct24) at each dilution, whereas any one of two target genes' positivity is considered as positive for this study.

Sensitivity of pooled specimen in various dilutions was analysed using any one target gene (n or ORFab1) positivity(Table 4). Sensitivity of pooled specimen decreases with increasing pool size. 100% sensitivity observed up to 10 pool and 15 pool sample in manual and automated extraction method respectively.

Table 4: Sensitivity percentage and 95% confidence interval of various polled sample both by manual and automated extraction method

Pool size	Manual extraction		Automated extraction	
	Sensitivity	**95%CI**	**Sensitivity**	**95% CI**
3P	100%	95.01% to 100%	100%	95.01% to 100%
5P	100%	95.01% to 100%	100%	95.01% to 100%
10P	99%%	92.5% to 99.96%	100%	95.01% to 100%
15P	81.94%	71.11% to 90.02%	99%	92.5% to 99.96%
20P	70.83%	58.93% to 80.95%	87.50%	77.59%to94,12%
25P	38.89%	27.62%to 51.11%	80.45%	69.53%to88.94%
30P	19.44%	11.06%to 30.47%	57.14%	44.75to 68.91%

Discussion

Pool testing in which multiple specimen is combined to test as a single specimen is not a new approach but previously used for screening donated blood for HIV. It has gained importance during COVID pandemic as it increases efficiency and output of surveillance where disease prevalence is low.

Previous studies exhibited good results in pools of up to 32 samples, and possibly even 64 samples, provided that additional PCR amplification cycles are done. [13]. To evaluate the effect of dilution, our study experimented with 3specimen, 5 specimen, 10 specimen, 15 specimen,20 specimen,25 specimen,30 specimen pool . Based on the comparative analysis of mean Ct difference between individual sample and pooled sample, it was evident that Ct value increases with dilution(Table 1and 2). Moreover, mean Ct difference was more obvious in manual extraction method than automated method in case of both target genes.Our findings of difference in Ct value are consistent with the study done by Abdalhamid et al.[12] Percentage of positive specimen at each Ct value was 100% in case of 5pool sample where extraction was done by both manual and automated extraction method(Table3). At higher Ct value (Ct 35),2positive sample out of 6 tested sample in pool became negative in 10 sample and 15sample pools, done by manual and automated extraction method respectively.It is different from the study done by Bateman et al who studied those pools of 5 and 10 become negative if the Ct value of initial positive specimen is between 30 and 35[14]. Another study has shown by Singh etal.(2020), increasing the number of positive samples maintaining the same pool size, subsequently increasing positivity ratealong with elevation of number of positive samples in a pool[15] Sensitivity markedly decreases(<90%) in20 pool and15 pool specimen in manual and automated extraction method respectively (Table 4). In a multi-site comparative study from India, they showed upto 100% sensitivity in 5 and 10 pool specimen upto Ct value 30, but sensitivity decreases in higher Ct values(>30) [16]. Dhibika et al.(2023) has depicted the RNA extraction process in automated method is more efficient for detection of SARS-COV-2 positive samples than the RNA extracted with manual kit-based method in case of E and ORF genes[17].

Although pandemic is over now but threats still loom large on the mankind as new variant ofCovid are emerging regularly. Experts allayed their concerns about the new variant called KP.2(FLiRT)which is spreading rapidly in some countries. In view of this we should enhance our surveillance by large specimen pool testing.

Conclusion

Pool testing is the best strategy to carry out population-wide surveillance as new variant of COVID-19 is coming up frequently. Our study indicates we can increase pool size upto15 specimen pool with minimal loss of sensitivity. Current study also observed sensitivity of automated extractor outweighs manual extraction method.

COVID-19 no longer constitutes a public health emergency of international concern but being a highly contagious disease, it has the potential to cause rapid outbreak especially in healthcare settings. So periodic surveillance of every ward and routine testing of every patient on admission should be incorporated inHospital infection control programme of every Health care setting. While in India we have a 5-sample pool testing strategy for surveillance as advised by ICMR, our study suggests we can increase pool size without compromising sensitivity up to 10-15 sample pool. Large pool testing will reduce cost and turnaround time while strengthen monitoring and add more power to Hospital infection control strategy which isthe key to reduce patient morbidity and mortality.

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