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Optimal size of Sample Pooling for RNA Pool Testing: An Avant-Garde for Scaling up Severe Acute Respiratory Syndrome Coronavirus-2 Testing

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Abstract

Background and Objectives: Timely diagnosis is essential for the containment of the disease and breaks in the chain of transmission of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). The present situation demands the countries to scale up their testing and design innovative strategies to conserve diagnostic kits and reagents. The pooling of samples saves time, workforce and most importantly diagnostic kits and reagents. In the present study, we tried to define the pool size that could be applied with acceptable confidence for testing. **Materials and Methods:** We used repeatedly tested positive clinical sample elutes having different levels of SARS CoV 2 RNA and negative sample elutes to prepare seven series of 11 pools each, having pool sizes ranging from 2 to 48 samples to estimate the optimal pool size. Each pool had one positive sample elute in different compositions. All the pools were tested by SARS CoV 2 reverse transcriptase quantitative polymerase chain reaction. **Results:** Out of the 77 pools, only 53 (68.8%) were found positive. The sensitivity of pools of 2–48 samples was decreased from 100% (95% confidence interval [CL]; 98.4–100) to 41.41% (95% CL; 34.9–48.1). The maximum size of the pool with acceptable sensitivity (>95%) was found to be of six samples. For the pool size of six samples, the sensitivity was 97.8% and the efficiency of pooling was 0.38. **Conclusions:** The pooling of samples is a practical way for scaling up testing and ultimately containing the further spread of the CoV disease 2019 pandemic.

Keywords: Coronavirus disease 2019, pool testing, optimal pooling, real-time polymerase chain reaction, severe acute respiratory syndrome coronavirus-2

Introduction

What started as a cluster of pneumonia cases in Wuhan city of China has become now a full-blown pandemic, the first of its kind due to the coronavirus (CoV).[1] This pandemic popularly known as CoV disease 2019 (COVID-19) is caused by severe acute respiratory syndrome CoV-2 (SARS-CoV-2).[2] As of 3rd June 2020, COVID-19 had globally caused 6, 287, 771 confirmed cases and 379, 941 deaths affecting over 200 countries.[3] Timely diagnosis is essential for the containment of the disease and breaks in the chain of transmission of SARS-CoV-2.

The laboratory diagnosis of SARS-CoV-2 is based primarily on nucleic acid amplification test (NAAT) such as real-time reverse transcriptase polymerase chain reaction (RT-qPCR). As the number of COVID-19 cases is increasing every day, the availability of diagnostic kits and reagents has emerged as a major bottleneck in the laboratory testing of SARS-CoV-2.[4] The currently available testing strategies mainly focus on symptomatic individuals. However, detecting the carrier or asymptomatic individuals holds the key in containing the spread of the infection into the community. Earlier the infected person is identified, sooner the spread of the infection can be

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contained and the surveillance machinery can be activated for contact tracing and ultimately break in the chain of transmission of the virus.[5] Densely and heavily populated countries such as India and other developing countries have abysmally low test rates per capita in the world,[6] and the number of cases is expected to shoot up post lockdown. Hence, the present situation demands countries to scale up their testing and design innovative strategies to conserve diagnostic kits and reagents.

The pooling of diagnostic tests, which has previously been applied in other infectious diseases,[7,8] is one such method where the samples are mixed and tested at a single pool and subsequent individual tests are made only if the pool tests positive. Pooling saves time, workforce, and most importantly diagnostic kits and reagents.

In the present study, we tried to define the pool size that could be applicable to a particular population with acceptable confidence and avoiding drop of positive cases which is a major risk of pool testing.

Materials and Methods

Sample collection

Combined nasopharyngeal and oropharyngeal swabs were collected and transported in viral transport media (VTM) maintaining the proper cold chain and sent to the virology laboratory of the Institute of Liver and biliary sciences, New Delhi, India. A volume of 200µl of the sample was further processed for viral nucleic acid extraction by Qiasymphony Diagnostic sample preparation (DSP) Virus/Pathogen mini kit (Qiagen GmbH, Germany) as per the manufacturer's protocol in elutes of 60 µl each.[9] Each sample was subjected to the addition of 10 µl of spiked extraction control at the time of extraction itself, to check the validity of the extraction procedure.

Performance of reverse transcriptase polymerase chain reaction in the laboratory

The 5 µl of the extracted RNA elute/sample was subjected to RT-qPCR for the qualitative detection of SARS-CoV-2 RNA utilising with AgPath-IDTM One-Step RT-PCR Reagents (Thermo Fisher Scientific) using an Applied biosystem (ABI) 7500 Real-Time PCR system (Thermo Fisher Scientific) and LightMix® SarbecoV E-gene (TIB MOLBIOL). Reactions were heated to 55°C for 5 min for reverse transcription, denatured in 95°C for 5 min, and then, 45 cycles of amplification were carried out for 95°C for 5 s and 60°C for 15 s using fluorescein amidite parameter for E gene. This assay targets the detection of the E gene for SARS as well as nCoV-2. The primer details are given in Table 1.

Primers and probes for the reverse transcriptase quantitative polymerase chain reaction

All samples that were screened positive for the E gene were confirmed by the performance of RT-qPCR for the detection of specific RdRp gene of SARS-CoV-2 using LightMix® Modular SARS-CoV-2 RdRP (TIB MOLBIOL) using similar PCR conditions as described above.[10]

Scheme of pooling of RNA elutes

Arbitrarily 7 twice tested SARS CoV 2 E and RdRp gene positive RNA elutes with varying cycle threshold values and 48 twice tested negative elutes for SARS CoV 2 E and RdRp gene tested by utilising AgPath-IDTM One-Step RT-PCR Reagents were selected. A total of 48 negative sample elutes were used to make eight series of 11 pools each (total of 77 pools) in an equal volume (3 µl each) of 2, 4, 6, 8, 10, 12, 16, 20, 24, 32 and 48 sample elutes. Each of the 11 pools was mixed with 1 positive elute to make its seven dilutions series of 1:2, 1:4, 1:6, 1:8, 1:10, 1:12, 1:16, 1:20, 1:24, 1:32 and 1:48 dilutions. A 5 µl of positive sample elute was mixed with the 5 µl of a pool of two samples to make 1:2 dilution, then this 1:2 mixture was serially double diluted to make 1:4, 1:8, 1:16 and 1:32 dilations using the pools of 4, 8, 18 and 32 sample elutes, respectively. A 3 µl of positive sample elute was mixed with the 15 µl of a pool of six samples to prepare 1:6 dilution of positive sample, then, this mixture was used to prepare serial double dilution of 1:12, 1:24, and 1:48 dilution using pools of 12, 24 and 48 samples, respectively. To prepare a dilution of 1:10, the 3 µl volume of positive sample was mixed with 27 µl of a pool of 10 samples, and then, it was double diluted with a pool of 20 samples to make 1:20 dilution of a positive sample. The cycle threshold of all the 7 positive samples used for serial dilution was 25, 29, 31, 33, 35, 38 and 39. A separate 8th dilution series of 11 pools was tested by adding PCR grade water instead of positive sample elutes which acted as a control. 5 µl of elute from the pool was used for RT-qPCR. Different 48 negative elutes were used to prepare the pools to determine the effect of the individual negative sample (to establish the heterogeneity) in the detection of a positive sample in the pool.

The frequency of Ct values distribution of individually tested positive samples reported from our laboratory during this pandemic was estimated and was categorised into ≤25, >25–29, >29–31, >31–33, >33–35, >35–38 and >38–40 ranges. The frequency of positive pools falling in these ranges was used to calculate the sensitivity of the pooling of sample elutes for RT-qPCR.

Known positive and negative sample elutes were used for making of pools, but for calculation, it was considered that if following a pooling algorithm after obtaining the results of pool testing, the same previously obtained results will get after individual testing of samples. The result of individually tested samples was considered the gold standard for the calculation of sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV).

Results

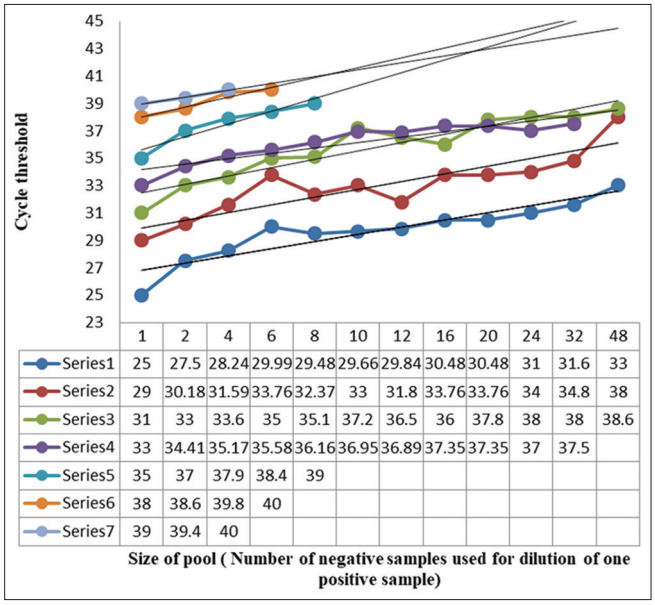
In this study, clinical samples previously established as positive for the SARS CoV-2 virus were chosen to determine whether they can detectable when their elutes mixed with negative

samples elutes in different dilutions. Positive sample elutes for SARS CoV-2 with a different range of Ct values of E gene target were chosen, serially diluted with negative sample elutes, and RT-qPCR was performed.

Overall, 88 pools were made which included 77 pools having one positive sample elute in different concentrations and 11 pools having no positive sample elutes. Out of the 77 pools of up to 48 times serial dilutions of positive samples, only 53 (68.8%) were found positive [Table 2].

Positive samples with Ct values ranging from 25 to 31 were detected in pools till 1: 48 dilution, while pools containing samples of Ct value 33 were detected till 1:32 dilution. Pools with higher Ct values became negative in lower dilutions. Sample with Ct values of 35, 38 and 39 was detected till 1:8, 1:6 and 1:4 dilutions, respectively. As the Ct value of an individual positive sample in the pool was increasing, the probability of detection of the positive pool was decreasing and as the size of the pool increased, Ct was attained later, as expected due to the dilution effect [Table 2 and Figure 1].

The frequency of Ct values (E gene only) distribution of individually tested samples was derived from 227 SARS CoV 2 E and RdRp gene positive samples detected from the 1st of March to 30th April 2020 using the same PCR reagents used for pool testing [Table 3].



	1	2	4	6	8	10	12	16	20	24	32	48
Series1	25	27.5	28.24	29.99	29.48	29.66	29.84	30.48	30.48	31	31.6	33
Series2	29	30.18	31.59	33.76	32.37	33	31.8	33.76	33.76	34	34.8	38
Series3	31	33	33.6	35	35.1	37.2	36.5	36	37.8	38	38	38.6
Series4	33	34.41	35.17	35.58	36.16	36.95	36.89	37.35	37.35	37	37.5	
Series5	35	37	37.9	38.4	39							
Series6	38	38.6	39.8	40								
Series7	39	39.4	40									

Figure 1: Ct values of pools having a positive sample of different Ct and trend of increase of Ct value as the pool size increases

Calculation of sensitivity of pool testing

Sensitivity of pool testing was defined as the probability that a true positive individual sample will be declared positive. The sensitivity of pooling was calculated by taking into consideration of the distribution of a particular Ct value in the results obtained during routine diagnostic testing of individual samples received. We defined seven ranges of Ct values and for pooling, we took samples with Ct value equal to the upper limit of the range as a more conservative approach [Table 3 and Figure 2]. All the pools of six samples having positive samples of Ct values 25–38 was tested positive, and the Ct values of 97.8% of individually positive samples during this pandemic were distributed within Ct value range of 25–38, and hence, the calculated sensitivity of the pool of six samples was 97.8% (95% confidence interval: 94.9–99.3). Similarly, the sensitivity of other pool sizes was calculated and shown in Table 2.

The sensitivity of pool testing decreased as the pool size increased and also as the Ct value of the positive sample in the pool increased. The acceptable sensitivity (>95%) of pool testing was found for pooling of up to six samples [Table 3 and Figure 3].

Calculation of specificity of pool testing

Specificity of pool testing was defined as the ability of the pooling algorithm to correctly identify those without the infection. For the calculation of specificity of pooling, a total of 11 pools of 2, 4, 6, 8, 10, 12, 16, 20, 24, 32 and 48 SARS CoV 2 E and RdRp gene negative samples

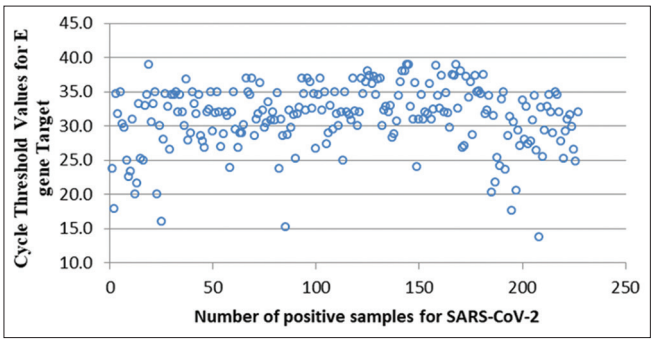


Figure 2: Cycle threshold values of 277 samples tested positive for E gene target of severe acute respiratory syndrome coronavirus-2

Table 1: Primers and probes for the real-time quantitative polymerase chain reaction

Gene	Oligonucleotide	Sequence
E gene	E_Sarbeco _F	ACAGGTACGTTAATAGTTAATAGCGT
	E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ
	E_Sarbeco_R	ATATTGCAGCAGTACGCACACA
RdRP gene	RdRp_SARSr-F	GTGARATGGTCATGTGTGGCGG
	RdRp_SARSr-P2	FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ
	RdRp_SARSr-R	CARATGTTAAASACACTATTAGCATA

FAM: 6-carboxyfluorescein; BBQ: Blackberry quencher

Table 2: Results, mean Ct values and clinical performance of pool testing

Pool series	Positive sample CT	2 sample	4 sample	6 sample	8 sample	10 sample	12 sample	16 sample	20 sample	24 sample	32 sample	48 sample
1	25	TD	TD	TD	TD	TD	TD	TD	TD	TD	TD	TD
2	29	TD	TD	TD	TD	TD	TD	TD	TD	TD	TD	TD
3	31	TD	TD	TD	TD	TD	TD	TD	TD	TD	TD	TD
4	33	TD	TD	TD	TD	TD	TD	TD	TD	TD	TD	TND
5	35	TD	TD	TD	TD	TND	TND	TND	TND	TND	TND	TND
6	38	TD	TD	TD	TND	TND	TND	TND	TND	TND	TND	TND
7	39	TD	TD	TND	TND	TND	TND	TND	TND	TND	TND	TND
Ct mean±SD	32.9±5	34.4±4.5	35.8±5	35.5±3.5	34.5±3.8	34.2±3.6	33.7±3.5	34.4±3	34.8±3.4	35±3.2	35.5±2.9	36.5±3
Water (control)		TND	TND	TND	TND	TND	TND	TND	TND	TND	TND	TND
Sensitivity % (95% CLs)		100 (98.4-100)	100 (98.4-100)	97.80 (94.9-99.3)	84.58 (79.2-89)	64.76 (58.2-70.9)	64.76 (58.2-70.9)	64.76 (58.2-70.9)	64.76 (58.2-70.9)	64.76 (58.2-70.9)	64.76 (58.2-70.9)	41.41 (34.9-48.1)
Specificity (%)							100					
PPV (%)							100					
NPV (%)		100	100	97.2	96.08	95.45	96.25	97.22	97.79	98.17	98.64	99.10

TD: Target detected, TND: Target not detected, PPV: Positive predictive value, NPV: Negative predictive value, CLs: Confidence limits, SD: Standard deviation

Table 3: Distribution of Ct value in positive samples received from 1st March to 30th April 2020 and Ct values of positive samples used in pooling and sensitivity of pools

Serial number	Distribution of Ct value in tested positive samples, $n=227$		Ct value of the Positive sample in pool	Maximum pool size detected positive	Sensitivity (95% CL) of pool testing
	Ct range	*n* (%)			
1	<25	23 (10.1)	25	48	41.41 (34.9-48.1)
2	25-29	37 (16.3)	29	48	
3	29-31	34 (15.0)	31	48	
4	31-33	53 (23.3)	33	32	64.76 (58.2-70.9)
5	33-35	45 (19.8)	35	8	84.58 (79.2-89)
6	35-38	30 (13.2)	38	6	97.80 (94.9-99.3)
7	>38	5 (2.2)	39	4	100 (98.4-100)
Median (IQR)	32 (34.7-29.0)		33 (36.5-30)		

CL: Confidence limit, IQR: Interquartile range

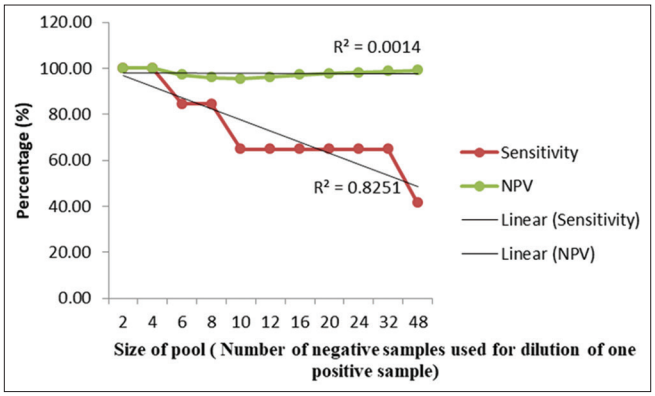


Figure 3: Sensitivity and negative predictive value of pools of different sizes. Figure shows the trend of sensitivity and negative predictive value of pooling as the pool size increased

elutes were tested. All the 11 pools tested negative and false-positive results were nil, and hence, the specificity of pooling of sample elutes for SARS-CoV-2 RT-qPCR was 100%.

Negative predictive value of pool testing

The NPV was defined as the probability that an individual specimen identified as negative at the end of a pooling algorithm was truly negative. The overall NPV was found to be 97.8%. The NPV for a pool size of 2 and 4 was 100% and found no false negative results, but for the pool sizes of 6, 8, and 10, NPV gradually decreased from 97.2% to 95.45% due to gradual increase in false negative results. For pool sizes from 12 to 48, NPV was progressively increased due to stable false-negative results but increasing pool size.

Positive predictive value of pool testing

The PPV was defined as the probability that a specimen identified as positive at the end of a pooling algorithm was truly positive. The PPV of pooling was 100%, and no false positive results were found.

Efficiency of sample pooling

The efficiency of a pooling algorithm was defined as the expected number of NAATs required per individual specimen evaluated. An efficiency of sample pooling was calculated 0.38 using the online calculator considering the two-stage Dorfman mini-pool strategy of pool testing with the conservative predictions of a sensitivity of 95%, a specificity of 99%, 4% prevalence of SARS-CoV-2 infection and pool size of 6 samples (http://www.bios.unc.edu/~mhudgens/optimal.pooling.b.htm).

Calculation of optimum pool size

Most positive pools reach the threshold at a later Ct as they are further diluted. A pool size of six samples having a sensitivity of 97.8% and NPV of 97.2% was considered acceptable for the pooling of sample elutes for RT-qPCR.

Discussion

While individual level NAAT remains the gold standard for the diagnosis of COVID-19 infection, a limited supply of diagnostic kits and reagents remains the major bottleneck in expediting testing of COVID-19 in the community especially during surge testing.[11] Furthermore, mass testing of samples should be done to control the COVID-19 pandemic at the earliest.[12] Hence, a testing method where a large number of samples can be tested and consuming minimal testing kits and reagent is the need of the hour. In this study, effect of pool size on the detection of SARS CoV 2 and its accuracy in the population was assessed. We found that a single clinical sample with SARS-CoV-2 RNA can be consistently detected in a pool of a maximum of four samples. The Ct values of individually tested RNA elute and that of the pooled group were in sync. As evident from Figure 1, pooling in this pattern leads to a gradual increase in the threshold cycle of the pooled group. Furthermore, additional amplification cycles could lower the detection limit allowing better detection of pools having samples of higher Ct values.[13]

The pooling of samples is essentially important in monitoring the infection in cohesive groups such as quarantine facilities, health-care workers, community surveillance and diagnosing the asymptomatic cases. The infection load in these groups may be low, but even a single positive case among such groups can activate the surveillance system and quarantine the affected group and prevent the further spread in the community. The pooling is better suited for low-prevalence populations, and it could fasten the testing over a very short time that would be able to identify new hotspot areas for infection before becoming condition worst. The pooling of samples can also be done before RNA extraction that is at the time of sample collection, putting the nasopharyngeal/oropharyngeal swabs into a common VTM and after sample collection by pooling of VTM. By doing so, the major bottleneck of RNA extraction can be removed.

The pooling of samples leads to the dilution of nucleic acid present in the pool leading to a decrease in the sensitivity. Hence, the pool size should be optimised for the agent to be detected and assay to be used so that even low-positive samples would not be missed in the pool testing. Gupta *et al*. showed that the increase in the cellular material, including nucleic acid, due to the pooling of multiple samples did not affect the detection of the SARS-CoV-2 virus by RT-qPCR.[14] The size of the pooling of samples should be such that it should give a positive result if it has at least one positive sample that has the lowest amount of viral nucleic acid so that the sensitivity does not get compromised. We determined the pool size by considering of amount of viral nucleic acid (as Ct value is usually proportional to the amount of nucleic acid) that is more prevalent in the community during this pandemic. A study by Arons *et al*. has shown that SARS-CoV-2 N1 targeted median Ct values for the four symptom status groups were similar (asymptomatic residents, 25.5; pre symptomatic residents, 23.1; residents with atypical symptoms, 24.2; and residents with typical symptoms, 24.8).[15] The median Ct value for samples tested positive in this pandemic was 32 (34.7-29.0). Nearly 97.8% of samples had Ct value of <38 for the 45 cycle protocol of RT-qPCR, and all the pools having a positive sample of Ct value <38 were tested positive up to the pool size of 6 samples, so we determined the optimal pool size of 6 for pool testing of SARS-CoV-2 RT-qPCR. The sensitivity (97.8%) and Negative predictive value (NPV) (97.22%) value of the pool of six samples were taken to consider it optimal pool size. The samples that had a Ct value of >38 were only 2.2%. The clinical significance of such low-positive samples could not be determined because of the unavailability of the gold standard viral culture methods.

The efficiency of individual testing is 1, and efficiency of <1 indicates that the pooling algorithm will require fewer tests on average than individual testing. By calculating on online calculator, we found the efficacy of pool testing of six samples 0.38, i.e., for one sample testing, only 0.38 test reagents are required (>95% sensitivity of the test, a prevalence rate of infection 4% and two-stage Dorfman mini-pool strategy used for calculation). Many pool testing strategies for pooling are known. The two-stage Dorfman mini-pool strategy is one of them which are more convenient for the pools of small sizes. In the first stage of this strategy, the master pool comprising all specimens is tested; if the master pool tests positive, all component individual specimens are tested (the second of the two stages).[16]

Conclusions

As the COVID-19 pandemic continues spreading, there is tremendous stress on logistics. Hence, pooling of samples is a practical way for scaling up testing and ultimately containing the further spread of the COVID-19 pandemic. This study suggests that a pool size of six samples would be optimal with acceptable confidence.

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Conflicts of interest

There are no conflicts of interest.

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