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Original Research Article

Evaluating the efficiency of specimen (sample) pooling for real-time PCR based diagnosis of COVID-19

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ABSTRACT

Purpose: This study is aims at evaluating the efficacy and sensitivity of specimen pooling for testing of SARS-CoV-2 virus to determine the accuracy, resource savings, and identification of borderline positive cases without impacting the accuracy of the testing.

Method: This study was conducted between August and October 2020, we performed COVID-19 testing by RT-PCR on the samples from varying prevalence of rural population (non-hot spot) referred to COVID laboratory, in the first step, the samples were collated into pools of 5 or 10. These pools were tested by RT-PCR. Negative pools were reported as negative whereas positive pools of 5 and 10 were then de-convoluted and each sample was tested individually.

Results: In the present study, we tested 1580 samples in 158 pools of 10 and 17,515 samples in 3503 pools of 5. Among 10 samples pool, 11 (13%) pools flagged positive in the first step. In the second step, among 11 pools (110 samples) de-convoluted strategy was followed in which 10 individual samples came positive. Among 5 samples pool, 164 (13%) pools flagged positive in the first step. In the second step, among 164 pools (820 samples) de-convoluted strategy was followed in which 171 individual samples came positive. The pooled sample testing strategy saves substantial resources and time during surge testing and enhanced pandemic surveillance. This approach requires around 76%–93% fewer tests in low to moderate prevalence settings and group sizes up to 5–10 in a population, compared to individual testing.

Conclusion: Pooled sample RT- PCR analysis strategies can save substantial resources and time for COVID-19 mass testing in comparison with individual testing without compromising the quality of outcome of the test. In particular, the pooled sample approach can facilitate mass screening in the early asymptomatic stages of COVID-19 infections.

1. Introduction

The novel coronavirus, COVID-19, was first reported and detected in Wuhan, China, in December 2019, and rapidly emerged as public health threat and spread throughout the world [1]. Thereafter, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the pathogen of coronavirus disease 2019 (COVID-19) has been declared a pandemic by WHO [2]. The Coronavirus Disease 2019 (COVID-19) pandemic has highlighted the need for early diagnosis of rapidly spreading infectious diseases for its better containment and eventual control. The ability to rapidly diagnose COVID-19 is important for evaluating the spread of disease and for tracing the contacts of infected individuals. There is ample proof that countries which are able to screen patients swiftly have fared better in containing the COVID-19 outbreak and reducing the mortality rate due to this disease [3]. Laboratory arrangements for testing for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been hampered due to the considerable strain on global supply chains for equipment reagents, personal protective equipment and other consumables. Many countries are experiencing an acute shortage of important reagents required for the polymerase chain reaction (PCR) assay for SARS-CoV-2 [4]. It is clearly understood that the rapid diagnosis of COVID-19 in both symptomatic and asymptomatic patients can shed light on transmission patterns and facilitate contact tracing for adopting a strategy for containment. Large scale population screening for COVID-19 infection is generally considered a necessary part advocated by WHO and ICMR.

The variable incubation period of COVID-19 infections of up to 14 days and an unknown number of asymptomatic carriers capable of

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transmitting the infection are big challenges for COVID-19 control and mitigation efforts. Mass testing is important for a wide range of further COVID-19 control strategies including checking for community transmission [5]. In addition, India has witnessed a unique issue of a huge migratory laborer population, moving in between states and metro cities to rural areas especially to states of Uttar Pradesh and Bihar. Confirmation of infection and its accurate diagnosis even in asymptomatic persons largely relies on real time reverse transcriptase PCR (rRT-PCR) tests [6]. Although rRT-PCR has been used in diagnostic and epidemiological studies for several other studies, the whole diagnostic process is laborious, time consuming and costly. Therefore, ICMR and several other research publications suggested the method of pool testing for establishing early diagnosis of large number of sample with optimization of cost and laboratory running time [7]. The concept of pool testing was given by Dorfman [8] in 1943 and has been earlier used for the detection of the human immune deficiency virus and hepatitis B/C viruses in blood products [9]. Key principles for successful application of group testing involve knowledge of the limit of detection, sensitivity and specificity of the assay, and the prevalence of the disease in the population [10]. The goal of the process is to determine the optimal pool size that provides the greatest conservation of resources while maintaining the reliable performance of testing. This study was thus performed for evaluating the efficiency of sample pooling for real time PCR based diagnosis of COVID-19. We also compared cycle threshold (Ct) values of positive pools with that of individual samples that tested positive upon deconvolution.

2. Materials and methods

The first pooling strategy we followed a simple two-stage testing algorithm known as Dorfman pooling. In the first stage, the samples are divided into disjoint pools of samples each, and each such pool is tested. A negative result implies that all samples in the pool are negative, while a positive result implies that at least one sample in the pool is positive. In the second stage, the samples of each pool that tested positive are individually tested.

To assess the group testing strategy, the first step was to calculate the most efficient pool size. Comprehensive epidemiological studies are still awaited to determine the prevalence of COVID-19 yet, it has been found to be wide spread and randomly distributed in large population throughout the country. However, the preliminary evaluation over a short period of time at our laboratory the observed specimen positive rate within the tested community had been within 2–3% for the past 5–7 weeks. Individual bar-coded samples were received at the laboratory. Based on the prevalence rate of 5%, we decided to perform pooled real-time reverse transcriptase polymerase chain reaction (rRT-PCR) testing and prepared 3505 pool of five samples and 158 pools of 10 samples each. The parameters and assumptions used in this calculation included an experimental assay lower limit of detection of 1–10 RNA copies/μl, an assay sensitivity of 95% or 100% and specificity of 100%. In the two-stage pooling algorithm, we divided the samples in pool sizes of 5–10 samples. We practiced the current testing procedure for diagnosing the presence of SARS-CoV-2 which begins with the collection of nasopharyngeal swabs and/or an oropharyngeal swab from the patient and transferring that sample in viral transport media (VTM). While preparing a pool of 5 and 10 samples, we pipetted out 50 μl and 25 μl samples each from all 5 VTMs and 10 VTMs, respectively, and transferred it to common eppendorf tubes (pool tube of 5 and pool tube of 10). About 560 μl of lysis buffer was added to the pool tubes and virus inactivation was done. RNA extraction was carried out using QIAmp RNA Mini Kit (Qiagen). rRT-PCR was performed using Lab Gun COVID-19 rRT-PCR Kit (Lab Genomics) as per kit protocol. Briefly, 20 μl reaction was prepared for the qualitative detection of SARS-CoV-2 RNA by qualitative rRT-PCR utilizing 5 μl of extracted RNA, 10 μl of two times PCR buffer, 1 μl of one-step enzyme, and 4 μl primers and probe mixture in two separate tubes for E genes and RdRp gene. Thermal cycling was performed at 55 °C for 30 min for reverse transcription, followed by 95 °C for 15 min and then 45 cycles of 95 °C for 15s, and 60 °C for 60s using Bio-Rad CFX-96. The threshold cycle (Ct) value for each well was calculated using the Bio-Rad cycler's software. It may be imperative to carefully choose the Cut-off Ct values for RT-PCR in evaluation of individual samples which for the study were taken as 36 for E-gene and RdRp. In order to arrive at a suitable cutoff value for both the pools of 5 and 10, various dilutions were studied. The sample dilution in duplicate were prepared at 1:1, 1:2, 1:4.1:8, 1:16 and the dilution of 1:3, 1:5, 1:10 was also evaluated for ascertaining the most suitable sample dilution. Four positive samples detected at different CT values (around 16, 24, 30, and 34) for both E-gene and RdRp were evaluated in the said dilutions. This provided an objective assessment of dilutions and Ct values for pool sample study. The trends in change of CT values were ascertained in the fore said dilutions. Further the positive samples after this rigorous evaluations were re-evaluated in dilutions of 1:3, 1:5, and 1:10. Thus, the average change in Ct values were +2.56 for pool of 5, and +3.38 for pool of 10 which was utilized in fixing the most suited cut off CT value of the pooled sample. Accordingly cut off.was raised for pool of samples of 5 and 10 by +3 and + 4 respectively. Hence CT value of <39 for sample pool of 5 and < 40 for pool of 10 was arrived and utilized in the study.

Samples negative in pool testing were reported as negative, whereas samples positive in pool testing were retested as a deconvoluted sample before finalizing the report. We also calculated the cost of consumables and kits used in the testing of pooled samples and deconvoluted samples for the estimation of the utilization of resources. The data generated in this prospective study was subjected to analysis with the help of appropriate statistical tools and for interpretation of significant outcome IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp was utilized.

3. Result

The study result showed that among 158 pools of 10 samples each, 11 pools flagged positive, whereas among 3503 pools of five samples each, 164 pools flagged positive. From 11 positive pools of 10 samples, 110 deconvoluted samples were tested and 10 (9%) individual samples were positive for COVID-19. In one pool of 10, with Ct 38.24 and 39.89 for E gene and RdRp gene respectively, we did not get any positive sample after deconvolution. Among 5 sample pool, 164 pools flagged positive which comprised of 820 samples. Among these deconvoluted 820 samples, 171 (20.8%) samples were positive (Table 1). In this study 181 samples were found positive in the total number of 19095 samples tested with a positivity rate of 0.95%.

To exclude false negatives, we selected random pools of 5 and 10 from regions showing higher positivity and tested individual samples after deconvolution. A total of 20 pools of 5, and 10 pools of 10 were selected on different days. We did not find any positive sample among these negative pools. In this study the pool testing of more than 10 samples were not evaluated for diagnostic purposes. Our study utilized a pool sample size of 5 for majority of samples for COVID-19 rRT-PCR testing for accuracy of diagnosis and easier deconvolution. Sample pool of 10 was discontinued for diagnostic purposes as it was difficult to handle, time consuming and moreover there were chances of missing the low positive samples.

3.1. Pooling as a resource-saving strategy

We have also evaluated pooling as a resource-saving strategy considering the prevalence of COVID-19 as 5% in our setting. Calculating resources which include consumables and kits for processing 100 samples while considering five samples to be positive (5% prevalence). We analyzed that compared with testing individual samples, we saved 60% consumables by using 10 sample pool strategies (Table 2). In this study, testing of 18837 samples required only 3601 tests to detect 181 positive individuals (prevalence 5%). These data suggest that pooling of up to 10

Table 1
Showing detailed pool results.

Total pools		Total samples	No. of Positive pools	No. of deconvoluted samples tested	No. of deconvoluted positive samples of positive pools
10 Sample pool	158	1580	11	110	10 (6.3%)
5 Sample pool	3503	17515	164	820	171 (5.3%)

Table 2
Tests consumables required for 100 samples using individual versus 10 sample pooled strategy.

Consumables required for individual samples	Consumables required in pool testing	Best possibility	Consumable required:
100		All 5 positive cases in single pool	20 tests(10pools+ 10 deconvoluted samples [1positive pool])
		Worst possibility All 5 positive cases in separate pool	Consumable required: 60 tests (10 pools +50 deconvoluted samples [5 positive pools])
Cost: 100%		Mean cost of pool testing: 40%	

Table 4
Table showing result analysis of borderline/low positive samples.

Pool with borderline positive sample (n = 6)					
S. no.	Deconvoluted Ct		Pool Ct		Pool detail
	E-gene	RdRp	E-gene	RdRp	
1.	29.12	35.81	32.37	38.64	Pool of 10
2.	28.42	32.48	31.48	35.26	Pool of 5
3.	31.32	34.94	36.64	38.47	Pool of 10
4.	29.84	35.42	33.51	38.82	Pool of 10
5.	30.48	36.09	34.28	39.56	Pool of 10
6.	30.14	35.37	34.09	37.48	Pool of 5

samples per pool can increase test capacity with existing equipment and test kits and detects positive samples with sufficient diagnostic accuracy. The above statement goes in favor of a pool of five samples also were we managed to save 90% of reagents kits, if we have tested individually all those 18837 samples it would have cost more than 20,000 reagents and kits and most importantly the diagnostic time because the testing capacity of our laboratory is 100–150 samples everyday but using two-step pool and deconvoluted strategy we increased our testing capacity maximum to 800 samples (average 700–900 samples every day).

3.2. Cut-off threshold results of pooled and deconvoluted samples

Results show that over a range of pool size, from 5 to 10 samples per pool. The average change in Ct value for E-gene were 3.70 and 2.56, and average change in Ct value for RdRp were 3.43 and 2.49 for pools of 10 and 5 respectively. Ct values were lower in deconvoluted positive individual samples compared with pools (Table 3).

3.3. Result analysis of borderline/low positive samples

In pooled strategy, somehow there are chances of missing the low positive cases as the viral load of low positive cases is diluted with increased volume in pooled samples but we have seen that these pools with atypical/low positive cases showed Ct value above 30, that is, 35.64–39.76, in most of the cases which on deconvoluted testing has lower Ct value around 30, that is, 26.12–32.28 (Table 4). We observed background signals carefully for low positive pools, and reactions with background signals were not interpreted as positive.

4. Discussion

The COVID-19 can present as asymptomatic or symptomatic infections. Earlier it was thought that viral loads in symptomatic patients are higher compared with asymptomatic cases. However, in a recent study, it has been documented that viral loads are similar in symptomatic and asymptomatic patients; thus, pool testing will produce similar results in all patients with COVID-19 [11]. The present study is concordant with the findings of Abdalhamid *et al.* [10] who reported that pool testing is effective in saving resources in the population having prevalence less than 10%. Laboratories have begun to demonstrate that SARS-CoV-2 can be detected in RT-qPCR performed on pooled samples, despite potential dilution. One limitation of pooling which authors feel is that positive sample reporting is delayed by a couple of hours which is taken in deconvoluting and retesting the specimen. They further concluded in their study of assessment of pooled testing to conserve resources that when the incidence rate of SARS-CoV-2 infection is 10% or less, group testing will result in the saving of reagents and personnel time with an overall increase in the testing capability of at least 69%. A recent study by Lohse *et al.* showed that over a range of pool sizes, from 4 to 30 samples per pool, Ct values of positive pools were between 22 and 29 for the envelope protein gene (E gene) assay and between 21 and 29 for the spike protein gene (S gene) assay. Ct values were lower in retested positive individual samples. The Ct values for both E gene and S gene assays in pools and individual positive samples were below 30 and easily categorized as positive. Ct value differences between pooled tests and individual positive samples (Ct pool − Ct positive sample) were in the range of up to five [12]. In another study on specimen pooling, it was observed that pooling did not affect the sensitivity of detecting SARS-CoV-2 when the PCR cycle threshold (Ct) of the original specimen was lower than 35. However, in specimens with low viral load (Ct > 35), 13.3% were false negative [13]. It can be explained by the fact that for each two-fold dilution Ct value increased by 1.24 [14]. In another study authors have performed pooling of RNA samples and observed similar results. [15].

ICMR has issued guidelines for expediting the COVID -19 testing using pooling of the samples and these guidelines clearly illustrates that pool testing may be deployed in the regions/area where positivity rate is below 2%. The positivity rates in the region from where the samples were evaluated in this study was <1% thus pool sample testing was deployed for optimizing the resources in the resource constrained set up.

Pool testing in the higher positive rate regions may not be suitable as it would give more number of positive pools which will not only affect the turnaround time (TAT) but require larger no of retesting. This would consume more resources. We adopted pool testing for this region (positivity <1%) and TAT for negative pools for samples up to 500 in the study was 8–9 h and, 11–12 h was attained for samples up to 1000. In our protocol the positive pools were evaluated with a TAT of 18–28 h after deconvolution.

It may be pertinent to add that Ct values inversely echo the viral load in the test sample. Lower Ct values in the sample are indicative of higher viral load which indirectly provides the measure of viral load in the

Table 3
Comparison of pool data between 10 sample versus 5 sample each pool.

Genes tested	Flagged pool	Average Ct change between flagged pools and deconvoluted positive samples
E-gene	Pool of 10	3.70
	Pool of 5	2.56
RdRp	Pool of 10	3.43
	Pool of 5	2.49

patients. However the Ct values are not absolute measure of the viral load. It is well established that Ct value directly depends on appropriateness of sample collections, storage and transportation of sample at suitable temperature. Quality and responsiveness of the kits used in nucleic acid extractions, RT-PCR and the sensitivity of the RT-PCR equipment used.

It may be clearly understood that higher the viral load in the COVID-19 patients, higher will be the viral shedding in the aerosols generated during coughing, sneezing and talking. These patients are categorized as highly transitive of the virus on contact and in the vicinity of their physical contacts. However, the chances of transmission of infection are independent of Ct values and patients testing positive for COVID-19 has always has the chance to transmit the infection.

To understand the advantages of a pooling approach, consider a laboratory receiving N = 100 samples and prevalence is 5%, that is, 5/100 samples are positive. If 10 pools are created for 100 samples then as a best-case scenario, we can have one pool positive and nine pools negative, and the total PCR reagent used to test 100 samples is 20. In the worst-case scenario, five pools will be positive thus total consumable used will be 60. So, taking the mean value as 40, we propose that for 100 samples in pooled testing we require 40 test reagents, thus saving 60% reagents. Each negative result obtained by a single RT-qPCR reaction determines that 100 individual samples are negative without the need for individual testing.

4.1. Limitations of the study

The study did not reveal any positive sample on deconvolution of the negative pools but it is possible if more number of negative pools would have been deconvoluted we could get borderline positive samples. The TAT of Individual positive samples from positive pools in our study was within of 28 h but TAT of positive pools may increase if the number of positive pools increases.

5. Conclusion

During a rapidly changing epidemic, testing strategies will need to adapt to potential increases in positivity rate. Group testing of pooled specimens also requires the use of highly sensitive assays to avoid missing low positive samples. Therefore, strategies must be employed to closely monitor the use of pooling as the positive rate of test specimens increases in an outbreak of disease. In addition, the impact of different extraction methods on the recovery of RNA and overall test sensitivity needs to be evaluated. Therefore, laboratories must perform their own validation pool studies for kits used for each RNA extraction and amplification based on the prevalence rate of COVID-19 in their own region. Finally, this study showed that pooling is an effective approach to expand the impact of limited test resources and reagents during specific stages of an infectious disease outbreak as it provides quality report in less time, cost, and man power.

CRediT authorship contribution statement

Surabhi Shukla: Conceptualization, Investigation, Resources, Writing – original draft, preparation. **Vandana Upadhyay:** Validation, Writing – review & editing, Supervision. **Vinod Kumar Maurya:** Methodology, Formal analysis, Resources, Writing – review & editing.

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