ORIGINAL ARTICLE

EFFECTIVENESS OF PCR POOL TESTING FOR SCREENING OF COVID-19 INFECTION IN PAKISTAN

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Background: We tested the utility of mini-pool PCR testing for the rational use of PCR consumables in screening for CoViD-19. **Methods:** After pilot experiments, 3-samples pool size was selected. One-step RT-PCR was performed. The samples in the mini-pool having COVID gene amplification were tested individually. **Results:** 1548 samples tested in 516 mini-pools resulted 396 mini-pools as negative and 120 as positive. Upon individual testing, 110 samples tested positive and 9 were inconclusive. 876 PCR reactions were performed to test 1548 samples, saving 43% PCR reagents. Centres with low prevalence resulted in most saving on reagents (50%), while centres with high prevalence resulted in more test reactions. Testing of individual samples resulted in delays in reporting. **Conclusion:** Pooling can increase lab capacity, however, pooling delays results and cause degradation of samples.

Keywords: Pooling; Pool testing; RT-PCR; COVID; SARS-CoV-2

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INTRODUCTION

The main strategy for prevention and containment of COVID-19 disease has been a three-pronged approach, commonly known as the 'test-trace-isolate' strategy. Tracing contacts with COVID-19 patients, and isolating lab-confirmed infections can prevent further spread of the disease in the community.[1,2] Countries have shown to reduce the number of new cases with massive population testing, tracing of contacts, and isolation of positive cases. This requires a massive expansion of testing capabilities. As many countries are experiencing shortages of diagnostic kits and the local industry is striving to keep up with the demand[3,4], it has become important to come up with new ways to conserve the reagents used for diagnostic tests. Confirmation of COVID-19 requires reverse-transcriptase polymerase chain reaction (RT-PCR) for the qualitative detection of the viral genome. Briefly, swab samples are obtained via the oropharynx or nasopharynx of the suspected patient in the community. These are then transported using an appropriate medium, the Universal transport medium (UTM) or Viral Transport Medium (VTM)) to the testing laboratory where total RNA is isolated, reverse transcribed into complementary DNA (cDNA) and amplified using probes and primers against the viral genes of SARS-CoV-2 causing COVID-19. This process is resource- and expert intensive requiring expensive equipment and reagents. Due to disrupted international trade, many of the consumables are not readily available. Therefore, novel approaches to testing are being utilized.

Sample pooling is a commonly practiced method in rare genetic disease research and blood plasma testing for detection of transfusion based transmitted infections.[5] Recently, pooling methods have been tried for COVID-19 testing as a way to test multiple samples in a single PCR assay.[6] In this method, swab samples can be pooled together in a batch of six or eight before transport and laboratory analysis – saving on sample collection tubes, RNA extraction and PCR reagents. Briefly, samples from multiple patients are pooled together (called a pool) and tested as one. If the PCR reaction on pooled sample shows no amplification (negative) for the disease-specific genes, all patients having their samples in that specific pool are labelled as negative. Whereas, if amplification is observed (positive), it implies one or more samples from that pool are positive. Individual patient samples are then tested separately to know which samples are positive. This can effectively decrease the number of tests needed.[7] Pool testing can be initiated at the community level or the laboratory level (Supplementary figures 1a and 1b respectively). The size of the pool is inversely proportional to the prevalence of the disease. The higher the prevalence, the smaller should be the size of the pool and vice-versa.[8] Sample pooling saves reagents and testing times. On the contrary, there are concerns over the dilution effects of pooling. Samples with very low level of the gene of interest might test false-negative in a pool due to the low limit of detection of the assay and an increase in turn-around time (TAT) of the result can result in sample degradation. The sample pooling

strategies like any other test urged to consider the pre-analytical, analytical and post-analytical phases of the laboratory process. Nevertheless, all the three phases are equally important in the good laboratory practices. COVID-19 sample pooling has been reported positively and effectively in the published literature. However, these reports have primarily focused on the analytical phase. The primary objective of the current study was to test the cost utility and effectiveness of COVID-19 screening in pooling as compared to individual sample testing. We asked whether sample pooling can be used to test more COVID-19 suspected samples while reducing the costs and TAT? Briefly, patient samples were tested individually as well with negative samples in pools of 3 (1:3) (one positive and two negative samples and so on), 5 (1:5), 7 (1:7), and 9 (1:9) and their results were compared.

MATERIAL AND METHODS

The ethical approval was granted by Office of Research, Innovation, and Commercialization (ORIC), Khyber Medical University (KMU). The study was conducted at Khyber Pakhtunkhwa Public Health Reference Laboratory (KP-PHRL), Peshawar, Pakistan, during April and May 2021. Suspected COVID-19 patients and their contacts were contacted by health-care workers. After detailed interviews and examination, nasopharyngeal swabs were obtained and transported in UTMs under a cold chain. Samples from various districts of Khyber Pakhtunkhwa province were received for COVID-19 PCR testing and included in this study. Since the current study is primarily focusing the analytical phase (testing and reducing cost), however, the pre- and post-analytical phases were also taken into consideration. In case of pre-analytical phase, all the samples were included only when i) the sample collection was performed within the last three days at the time of testing and ii) the samples follow our laboratory (PHRL) standard operating procedures (SOPs). Further, the quality of the graph for the IC was considered to assess quality that is actually meant to depict the quality of the specimen collected. The date of sample collection was checked in the online software and some major points of the SOPs were that specimen must be packed properly in a maintained cold chain with no visible leakage. For the post-analytical phase in COVID-19, we have focused the TAT of generating the reports of the tested samples.

First, to assess the effect of dilution factor due to pooling on results, a pilot experiment was performed. Based on Cycle Threshold (Ct), we first identified three strong positives (<25 Ct), three medium positives (Ct of 26–32), and three weak positive (Ct of 33-37) (Figure-1). Subsequently, each positive sample was diluted with negative samples in the ratio of 1:3, 1:5, 1:7 and 1:9. RNA was extracted and PCR was performed according to the manufacturer's protocol. Based on the results of the 1st phase, we divided 140 samples into individual pools of 5 in the next phase. A negative outcome meant that all results were negative in the pool, and a positive result means that at least one sample was positive in the pool. In the second level, the samples from the positive pool were tested individually. Pooling was validated on an individual cohort of samples in mini-pools of 3 from different centers. The results were interpreted according to the manufacturer's specifications (Table-3).

200 µl of each sample was taken into a new tube, mixed thoroughly and a total of 200µl sample from the fresh tube was used for extraction according to the manufacturer protocol. RNA was extracted using Ascend Magnetic Bead RNA extraction Kit (cat # AS001 Luoyang Ascend biotechnology China) using the Hero 32 RNA extraction system (Model No. AS90334, Luoyang Ascend biotechnology China) using manufacturer's guidelines.

Multiplex one-step reverse transcriptase amplification approach was used for detection. Nucleic Acid Diagnostic Kit by Sansure Biotech (Sansure Biotech Inc China, Ref No. S3102E) was used for amplification. The kit utilizes novel coronavirus (2019-nCoV) *ORF1ab* and Nucleocapsid (N*)* gene as the target regions in addition to the human RNase P as an Internal Control (IC). Thermal cycling conditions, reaction composition and result interpretation were performed as per the manufacturer's instruction (Supplementary Table1) on ABI 7500 (Applied Biosystems™ 7500 Real-Time PCR Instrument (7500 Software v2.3 Cat. No: 4351105).

RESULTS

First, 9 positive samples were selected purposively all with good internal controls (ICs): strong positives (viral genes ct <25), moderately positive (viral gene ct 26-32), and weakly positive (viral genes ct 33–37), 3 samples each. Each sample was pooled with negative samples in pools of 3, 5, 7, and 9. RNA extraction and PCR reactions were performed and analyzed. Amplifications were seen in all pools. However, in pools of 7 and 9 dilution effect was observed. N and *ORF1ab* genes did not amplify in 1 pool of 7 and 9 respectively (representative PCR amplification plots are shown in figure 1).

Next, a total of 140 random samples from different sampling centres were obtained and pooled in 28 pools of 5 samples each. RNA extraction and RT-PCR were performed for the pools as well as individual samples. PCR assays were analyzed and reported. A total of 14 pools tested negative and 14 pools tested positive as mentioned in Table 1. In

parallel, these samples were analysed individually. Of 70 samples from the 14 positive pools, 31 were positive, 35 negative, and 4 samples had no amplification in the internal control gene. In 70 samples from 14 negative pools, 68 samples were negative whereas 2 samples showed no amplification in the internal control genes. Out of 28 pools, 26 pools had concordance with their individual samples' PCR assays, i.e., positive pools had at least one positive sample and negative pools had negative samples. However, in one negative pool, 2 samples showed no amplification in internal controls, implying these samples had a poor quality sample and the suspected patient needed to be sampled again. In positive pools with a single positive sample, the Ct values of *N* or *ORF1ab* genes showed delayed amplification by 1–2 CT cycles as compared to the individual sample's assays. The rate of positivity was 50% in pools and 22% in the entire cohort of samples tested individually.

Next, we tested the validity of mini-pools in an independent cohort of samples. Mini-pools of 3 instead of 5 were tested to increase the sensitivity of the assay. We tested samples from four COVID-19 quarantine centers of varying patient burden. In 1191 samples from center 1, 334/397 mini-pools tested negative while 63 pools showed amplification and were tested individually in 189 PCR reactions. Out of these, 38 samples showed positive amplification. A total of 586 tests were conducted to screen 1191 samples resulting in 51% fewer tests conducted. In center 2, 24/35 pools tested negative, whereas 11 pools had to be tested individually. A total of 68 PCR reactions had to be conducted to screen 105 samples resulting in 35% fewer tests. In the third cohort, 183 samples were pooled into 61 pools. Out of these, 19 pools were negative, and 42 pools tested either positive or inconclusive. These were tested individually. A total of 187 PCR reactions had to be performed to test 183 samples resulting in 2% more PCR reactions performed than would have been required. In the fourth cohort, 69 samples were pooled in 23 pools. Four pools tested positive and a total of 35 reactions were performed, saving the cost for 49% reagents. Overall, 1548 samples were tested in 516 pools and a total of 876 tests were performed with 43% less consumption PCR reagents. These results are summarized in Table 2. All the results of negative pools were issued within 24 hours. However, all the individual results from positive pools could not be finalized within the 24 hours duration. Notably, 9 samples from 120 positive pools (360 samples) showed no amplification in internal control genes.

Table-1: Pool testing results versus individual samples' results

Legend: - means 'negative' (no amplification in CoViD-19 specific genes), + means positive (amplification in CoViD-19 specific primers), 0 means 'no amplification in internal control and CoViD-19 specific genes). Pool results were considered 'concordant' if results of pooled samples showed amplification and there was at least one positive individual sample. These were considered discordant when results of a pool showed no amplification but one or more of individual samples either showed amplification in CoViD-19 specific genes, or, no amplification in internal control genes.

Pool no	Pool result	Individual samples' results	Concordance
1.	-	-----	Concordant
2.	-	-----	Concordant
3.	-	-----	Concordant
4.	-	-----	Concordant
5.	+	-+---	Concordant
6.	+	+++-+	Concordant
7.	+	+-+--	Concordant
8.	+	++0--	Concordant
9.	-	-----	Concordant
10.	-	-----	Concordant
11.	+	--+-+	Concordant
12.	+	+-+--	Concordant
13.	+	+----	Concordant
14.	-	-----	Concordant
15.	+	0++--	Concordant
16.	-	-----	Concordant
17.	+	--++-	Concordant
18.	-	-----	Concordant
19.	+	+-+++	Concordant
20.	-	-----	Concordant
21.	+	-+-+-	Concordant
22.	-	----0	**Discordant**
23.	+	---+-	Concordant
24.	+	0+---	Concordant
25.	+	--+-+	Concordant
26.	-	0----	**Discordant**
27.	+	-+--+	Concordant
28.	+	++---	Concordant

Table-2: Pooling of patients from four different cohorts, with different TPR% and percent reagents saved

Cohort	No. of samples	No. of pools	Negative pools	Positive pools	Positive samples	No IC*	Tests performed	TPR#	Tests reagents saved
Centre 1	1191	397	334	63	38	5	586	3.19%	50.8%
Centre 2	105	35	24	11	17	2	68	16.19%	35.2%
Centre 3	183	61	19	42	51	1	187	27.87%	-2.2%
Centre 4	69	23	19	4	4	1	35	5.80%	49.3%
Total	**1548**	**516**	**396**	**120**	**110**	**9**	**876**	**7.10%**	**43.4%**

* No IC= no amplification in the internal control gene, #TPR: Test positivity ratio.

Table-3: **Results analysis according to the manufacturer's instructions**

	Reaction condition	Interpretation
1.	Amplification in the internal control gene <40 Ct cycles	Successful amplification/adequate sample
2.	No amplification in the internal control gene	Failed amplification/inadequate sample
3.	Amplification in either *N*- or *ORF* gene with Ct value <37	Positive for COVID-19 virus
4.	Amplification in either *N*- or *ORF* gene with Ct value between 37-40	Inconclusive. Repeat required
5.	No amplification in either *N*- or *ORF* gene	Negative for COVID-19 virus

DISCUSSION

We tested the real-life utility of laboratory-based pooling of nasopharyngeal swabs from suspected COVID-19 patients. We demonstrate that the dilution effect is minimal with a pool size of up to 5 samples. Pooling samples together give concordant results in the majority of pools. Also, we showed that reagent's cost can be saved up to 50% in centers where TPR is 3–5%. However, pooling masks degraded samples within a pool. Another issue with pooling is delayed turn-around time for positive samples and degradation of samples because of repeat sampling.

Li H *et al*,[9] also successfully applied the 10:1 pooling strategy in a single center. The viral copy numbers in a nasopharyngeal swab from a patient remain high during the symptomatic phase of the disease, peaking $7.1x10^8$ copies per nasopharyngeal swab.[6] The dilution effect in pool sizes of up to 10 is unlikely to affect the results.[10] Indeed, some mathematical models have suggested a pool size of up to 94.[8] Hirotsu Y *et al*, demonstrated the applicability of the pooling technique to screen large cohorts of hospital healthcare staff and patients.[11] We find the dilution effect in pool sizes of 7 and 9 samples. The reason for this difference may be pool cold-chain maintenance in the hot climate of Pakistan. We also find that coherent to other studies, pooling multiple specimens together results in a mild - but anticipated - decrease in PCR cycle thresholds.[12] As pool size increases, according to Yelin I *et al*, each respective sample and possible SARS-CoV-2 RNA is diluted, leading to an observed linear Ct increase of 1.24 for every double dilution.[13] The majority of statistical estimates predict that pools of 4–5 samples maximize assay benefits by reducing the false-negative rate and preserving performance.[14–16] The FDA recent report showed that four swabs can be effectively pooled together in emergencies (https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-issues-first-emergency-authorization-sample-pooling-diagnostic) which has a comparable dilution effect to ours. Timo De Wolff T *et al*, also used this approach in COVID-19 and concluded that pooling increases the throughput of the test and saves time.[17] In addition, Watkins AE *et al*, support the pooling strategy which is beneficial for ongoing surveillance.[18] The optimum size of the pool depends on the sensitivity and specificity of the PCR kit as well as the prevalence of the disease.

COVID-19 has a short disease course, the exact prevalence cannot be calculated in most settings. Instead, Test Positivity Ratio (TPR) is used to estimate the incidence of disease. Bukhari *et al*[19], in a simulation exercise, demonstrated that with a current TPR of 8.6, a pool size of 4–8 would use about 48% fewer PCR kits. However, mathematical models assume a uniform prevalence of the disease in a community. This assumption could be misleading as the labs receive samples from different populations with varying levels of TPR. In our study, we observed a great diversity of TPR between different cohorts of samples. The fact that TPR varied between 3.19–27.8% from within the same city, meaning that accurate assessment of pool size cannot be made. Two of the sample cohorts tested were from the international borders. With passengers arriving from various countries, the estimation of pool size is not possible. Furthermore, even within a community with known TPR, clusters of the high and low prevalence of disease might exist.

COVID-19 testing relies on rapid turn-around time for effective public health response such as isolating positive cases from the rest of the community. In sample pooling, positive pools need to be tested individually – thereby increasing the turn-around time for positive patients. This has grave consequences for outbreak response thus compromising the post-analytical phase of the laboratory testing. With the aim of disease

containment and prevention of spread, delay in reporting is not justified. However, when the disease prevalence crosses the threshold for containment, and mitigation strategies are adopted, the aims of widespread testing is restricted to estimating the population disease trends.[20] Another notable finding in our study was of misdiagnosis of inadequate samples as negative. Due to the lack of adequate capacity for sample taking, transport and shipment, inadequate samples may be sent for testing. Such inadequate samples show no amplification of the internal control gene. However, in pools, internal controls are always amplified and such inadequate samples are masked. Although a small proportion of the total number of samples, this is potentially an error that needs to be considered before adopting pooling strategies.

Therefore, the pooling of samples may increase the testing capacity of resource-constrained health-care systems, have the following limitations:

First, public health and laboratory scientists in Pakistan have shown interest in exploring sample pooling as a way to reduce costs. However, in these very settings, limited data available on the prevalence of the disease in various population groups. Pooling strategies are cost-effective only when the prevalence of the disease is low. Secondly, effectiveness of pooling targets only reducing PCR reagents - which forms a small proportion of the overall resources required in the analytical phase of a test development. The entire system for the pre-analytical phase like sample collection, transportation, entry of patient details, and the post-analytical phase such as reporting, and report communication remains the same. The reporting of positive samples could be even delayed which remains a standalone major drawback of COVID-19 pooling. Therefore, cost-saving is a very small proportion of the entire cost of the testing mechanism. Thirdly, pooling may result in delays in reporting. In our experiments, reports of negative samples were delivered to the healthcare team and the patients within the expected turn-around time. However, samples from positive pools – nearly half of the total pools - had to be tested individually. In COVID-19, rapid reporting and response are crucial for disease containment. A delay of up to 12 hours due to repeat testing may have significant adverse effects on the public health response in general and the individual patient in particular. Taken together, our findings suggest that sample pooling for COVID-19 testing, can only be permitted during disease mitigation stages. These should not be used for diagnosis of individual patients.

In summary, we show that sample pooling saves PCR reagents and consumables when the prevalence of the disease is low and the required sensitivity can be obtained with 1:3 pool size. Furthermore, saving is less when the prevalence of the disease is high as positive pools have to be tested individually.

Pool testing in a laboratory setting is a valid method to improve testing capacity and reducing cost in the analytical phase (PCR testing). However, two major points should be considered. This method should be carried out in selected cohorts with a low prevalence of the disease to control the potential pre-analytical biases of sample degradation or compromised sample collection. Alternately, the size of the pool may be reduced to 1:3 if the suspected prevalence of the disease is at or higher than 3%.

Conflict of Interest:

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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AUTHORS' CONTRIBUTION

NA: Performed research, analysed data, wrote manuscript. Azra: Performed research, analysed data, wrote manuscript. HM: Performed research, analysed data. INK: Performed research, analysed data, wrote manuscript. SS: Analysed data, wrote manuscript. SA: Performed research, analysed data. MZ: Performed research, analysed data, wrote manuscript. AA: Conceived research, designed methodology. YMY: Conceived research, designed methodology, analysed data, and wrote manuscript. All authors approved the final draft of manuscript.

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