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Feasibility, efficiency & effectiveness of pooled sample testing strategy (pooled NAAT) for molecular testing of COVID-19

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***Background & objectives*: During the current COVID-19 pandemic, a large number of clinical samples were tested by real-time PCR. Pooling the clinical samples before testing can be a good cost-saving and rapid alternative for screening large populations. The aim of this study was to compare the performance characteristics, feasibility and effectiveness of pooling nasal swab and throat swab samples for screening and diagnosis of SARS-CoV-2.**

***Methods*: The pool testing was applied on a set of samples coming from low COVID-19 positivity areas. A total of 2410 samples were tested in pools of five samples each. A total of five pools of five samples each were generated and tested for *E* gene.**

***Results*: Of the total of 482 pools (2410 samples) 24 pools flagged positive. Later on pool de-convolution, a total of 26 samples were detected as positive for COVID-19, leading to positivity of about one per cent in the test population. For the diagnosis of individual samples, the pooling strategies resulted in cost savings of 75 per cent (5 samples per pool).**

***Interpretation & conclusions*: It was observed that testing samples for COVID-19 by reverse transcription (RT)- PCR after pooling could be a cost-effective method which would save both in manpower and cost especially for resource-poor countries and at a time when test kits were short in supply.**

Key words COVID-19 - cost-effective testing - diagnostics - pool testing - reverse transcription-polymerase chain reaction - sensitive - specific

In a pandemic situation there is a need to test a large number of samples for virus detection. It is not a possibility to expand laboratory capacity exponentially. Moreover, in low- and middle-income countries detection using real-time PCR technologies may not be cost-effective for testing each individual. Many of the testing centres lack automation hence require large number of trained manpower. Pooling samples can be an efficient way to screen for the nucleic acids of viruses, bacteria or parasites[1,2]. A pooled testing

algorithm involves the PCR screening of a specimen pool comprising multiple individual patient specimens, followed by individual testing (pool de-convolution) only if a pool flags positive[3]. As all individual samples in a negative pool are regarded as negative it results in substantial cost savings when large number of pools test negative.

This study was planned to demonstrate the feasibility of sample pooling for PCR screening for COVID-19 and to assess the efficiency and effectiveness of sample pooling in COVID-19 testing using reverse transcription (RT)-PCR.

Material & Methods

The study was conducted in the virology laboratory, department of Microbiology, King George's Medical University, Lucknow, India, during March 25 and April 10, 2020 on COVID-19 nasal swab and throat swab (NS/TS) samples. The study protocol was approved by the Institutional Ethics Committee. To determine the analytical sensitivity of the pooled and non-pooled samples different panels of clinical samples were used.

Statistical feasibility (pool size estimation): Statistically probabilities of optimized batch sizes (b) were worked out considering dynamic conditions of rapidly increasing numbers for two types of pooling (repeated pooling and one-time pooling) based on total expected samples (N) and frequency of positive samples (in the absence of population prevalence (p)[2]. One-time pooling was considered due to simplicity as shown in Table I. Statistically optimized batch size was 64 (max)[2-4]. Theoretically, this was acceptable for monitoring of positivity among pools when large scale screening was intended, especially for surveillance purpose. Since this was for diagnosing positive cases, it was considered that no positive case should be missed because of dilution effect. For pooling with de-convolution, a maximum of 10 sample pooling was found appropriate based on the biological plausibility (of retaining same characteristic from 25-30 µl into the pooled sample of 250 µl) based on criteria for Dorfman procedure[4] for adequacy of individual samples characteristics and use of micro-pipette for subsequent testing retaining test accuracy[2,5]. However, for the present study, it was decided to pool only five samples/ test, so that no positive case was missed.

Table I. Statistical feasibility of samples in one-time pooling

Range of *P*	Range of ratios of positive samples	Optimal sample batch size	Fraction of tests needed
0.04<*P*<0.2	<1:5	4	0.40-0.84
0.008<*P*<0.04	<1:25	8	0.19-0.40
0.003<*P*<0.008	<1:125	16	0.11-0.18
0.001<*P*<0.003	<1:333	24	0.07-0.11
0.0005<*P*<0.001	<1:1000	32	0.05-0.06
P<0.0005	<1:2000	64	<0.05

Biological plausibility and experimental feasibility assessment: Considering sample quantity of 250 µl required for PCR testing, it was decided to pool five samples so that 50 µl sample could be taken from each for the pool. The stored COVID-19 positive samples with different cyclic threshold (Ct) value range were taken from the repository stored at -80°C. All the samples had a unique identification number and were recorded in such a manner that individuals could not be identified, directly or through identifiers. A total of 50 µl each of one positive sample and four negatives samples were mixed in a single tube.

Throat swab/nasal swab (*TS/NS) pool constitution*

Pool one: Five pools of five sample each containing four negative and one positive specimen which was tested positive (Ct >15-20).

Pool two: Five pools of five sample each containing four negative and one positive specimen which was tested positive (Ct >20-25).

Pool three: Five pools of five sample each containing four negative and one positive specimen which was tested positive (Ct >25-30).

Pool four: Five pools of five sample each containing four negative and one positive specimen which was tested positive (Ct >30-35).

Pool five: Five pools of five sample each all of which were tested negative.

These pools were tested by real-time PCR for the *E* gene as per ICMR-NIV, Pune, SOP instructions[6]. Testing was conducted in triplicates and the average of three runs was used as Ct for the analysis. All

the positive and negative samples used for pool preparation were tested in parallel with pooled samples to make sure that the samples were not degraded during storage.

Real-life application with post-facto efficiency and effectiveness: The sample pooling technique in routine testing was done as per guidelines in ICMR COVID-19 sample pooling advisory[7]. Pools were created using 50 µl of NS/TS specimen from each one of five samples for a final volume of 250 µl. The whole volume was used for RNA extraction. Samples were chosen either from those geographical areas where the positivity rate was negligible or from areas surrounding the hot spots, consecutively. Samples coming from hot spots were not used for pool testing.

Viral nucleic acid extraction: Total viral nucleic acid was extracted from whole 250 µl of NS/TS sample using the PureLinkViral DNA/RNA mini kit (Invitrogen, Carlsbad, USA) as per the manufacturer's instructions. Viral nucleic acids were eluted from the filter column with 50 µl of nuclease-free double distilled water and stored at -80 °C until further use.

Real time PCR and interpretation of results: TaqMan real-time PCR for testing the presence of SARS-CoV-2 was used as per the WHO protocol using SuperScriptIII Platinum One-Step quantitative RT-PCR (Invitrogen, Carlsbad, USA) master mix[8]. All the samples went through testing protocol consisting of first-line screening which included *E* gene (for coronavirus) and *RnaseP* (human housekeeping control/internal control) gene. If the sample was positive for the *E* gene then confirmatory assay was carried out for *ORF* and *RdRp* gene targets. The real-time PCR sensitivity, in terms of 95% hit rate was about 5.2 RNA copies/reaction (at 95% hit rate; 95% confidence interval: 3.7-9.6 RNA copies/reaction could be detected)[9].

Applicability, advantages, and disadvantages of pooled and non-pooled sample testing: An analysis was conducted to understand the applicability, advantages and disadvantages of pooled testing in screening for COVID-19. The analysis was conducted in terms of the difference of sensitivity among both the methods and the cost saved in testing samples in pools[10].

Results & Discussion

Biological plausibility and experimental feasibility assessment: Ct values of *E* gene both in pooled sample tests and individual sample tests are mentioned in Table II. Results were comparable in pools and individual sample test (100% sensitive). All the pools containing the positive samples tested positive. Ct values of individual sample and pool were comparable with maximum Ct difference of 1.2 while the average deviation was ±0.7 Ct. In pool 4 with Ct ranging 30-35 one pool showed positive reaction in two of the three times. It showed that five sample pooling was biologically plausible and technically feasible for PCR testing of COVID-19.

Real-life application with post-facto efficiency and effectiveness: The results of the 482 pools of samples conducted in 21 different sets showed encouraging results. Of the 482 pools tested, 24 pools were flagged as positive. Set six containing 24 pools (120 samples) had 12 pools flagged which was an outlier (Table III). In this set with a positivity of >10 per cent we could save 30 per cent tests by pooling the samples. Overall, the efficiency in terms of the number of tests was increased by 300 per cent in almost all sets. After adjusting for time, workforce and logistics required for sample pooling and de-convolution, the efficiency of sample pooling was retained.

A total of 26 samples (2 pools tested COVID-19 positive for 2 samples each) were tested positive out of the 24 pools flagged as positive. Of the 24 flagged pools, 26 samples were positive for COVID-19 RNA as two pools contained more than one positive COVID-19 samples. The comparative analysis of the cost difference in samples tested individually and in pools of five showed that the pool testing reduced the requirement of the reagents to 1/4th saving up to 75 per cent of the cost involved in testing. Pooled sample testing and testing showed 100 per cent sensitivity (Table IV).

In this study, it was observed that testing samples for COVID-19 by RT-PCR after pooling might be a cost-effective method which would save both in terms of workforce and cost. The strategic pooling of NS/TS samples will help in bulk increase of testing capacity and cost reduction of RT-PCR testing during the COVID-19 pandemic. The method retained accuracy

Table II. Comparison of cyclic threshold values of *E* gene by individual and pool testing

Average Ct of positive sample when tested individually (250 µl sample)	Average Ct of positive sample when tested in pool of five (250 µl sample)	Difference in Ct
Pool 1: Sample Ct range (15-20)		
14.89	14.45	0.44
14.04	14.48	0.44
13.47	14.21	0.74
20.49	21.52	1.03
17.97	19.03	1.06
Pool 2: Sample Ct range (20-25)		
21.11	20.48	0.63
24.42	25.52	1.1
24.53	23.79	0.74
22.39	23.61	1.22
25.12	25.29	0.17
Pool 3: Sample Ct range (25-30)		
26.12	25.89	0.23
27.44	28.34	0.9
28.76	29.45	0.69
26.47	27.17	0.7
30.92	32.11	1.19
Pool 4: Sample Ct range (30-35)		
33.47	34.51	1.04
32.29	33.12	0.83
34.94	36.12	1.18
33.25	34.21	0.96
35.49	36.68	1.19
Pool 5: Negative samples		
ND	ND	ND
ND	ND	ND
ND	ND	ND
ND	ND	ND
ND	ND	ND

ND, not detected; Ct, cyclic threshold

of the test. The sensitivity of conventional individual sample testing was retained.

The pool testing was applied on the set of cases coming from low COVID-19 positivity areas. Only one set of the pool (set 6) containing 24 pools showed high flagging rate (12/24 flagged). This was due to the emergence of a new hot spot in one of the districts. Of the total of 482 pools (2410 samples), 24 pools were flagged. A total of 26 samples were detected as positive for COVID-19, leading to a positivity of approximately one per cent among the study population.

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Table III. Results of pooled testing and the number of flagged pools

Pool sets	Number of pools tested in set	Number of samples tested in pool	Flagged positive pools	De-convolution tests	Total number of PCR tests if 5 pooling (with de-convolution of positive pools)	Proportion of tests saved (%)
Set 1	13	65	0	0	13	80
Set 2	31	155	2	10	41	74
Set 3	12	60	0	0	12	80
Set 4	3	15	0	0	3	80
Set 5	8	40	0	0	8	80
Set 6*	24	120	12	60	84	30
Set 7	24	120	1	5	29	76
Set 8	16	80	0	0	16	80
Set 9	30	150	1	5	35	77
Set 10	6	30	0	0	6	80
Set 11	14	70	1	5	19	73
Set 12	31	155	1	5	36	77
Set 13	17	85	0	0	17	80
Set 14	9	45	0	0	9	80
Set 15	48	240	0	0	48	80
Set 16	43	215	1	5	48	78
Set 17	4	20	0	0	4	80
Set 18	22	110	0	0	22	80
Set 19	34	170	1	5	39	77
Set 20	46	230	4	20	66	71
Set 21	47	235	0	0	47	80
Total	482	2410	24	120	602	75

*Emergence of new hot spot

Table IV. Comparison of expenses done if samples are tested individually versus in pools of five

Parameter	Samples tested individually	Samples in pools of 5
Individual samples or pools tested (n)	2410	482
Positive pools (n)	-	24
Samples found positive	26	24
Sensitivity of pooled testing (%)	NA (reference value)	100
Total cost in %	Actual cost (X)	24.86 of X
Total cost savings (%)	Actual saving (Y)	75.23 of Y

NA, not available

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***Conflicts of Interest*:** None.

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