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Original Article

Evaluation of detection of severe acute respiratory syndrome coronavirus-2 by chip-based real-time polymerase chain reaction test (truenat™ beta CoV) in multi-sample pools

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Abstract

Introduction: Systematic testing for Severe Acute Respiratory Syndrome CoronaVirus-2 (SARS-CoV-2) using molecular diagnostic tools to identify individuals with coronavirus disease 2019 (COVID-19) infection, and tracing their primary and secondary contacts is important to curb its spread. With resource limitations on testing individual samples, testing of pooled samples provides alternative approach to increase testing capacity. Present aimed at assessing the detection of SARS-CoV-2 RNA in pooled samples using chip-based real-time polymerase chain reaction Test (Truenat™ Beta CoV).

Materials and Methods: Pooled sample size of five was used from laboratory confirmed COVID-19 positive and negative samples. SARS-CoV-2 positive nasopharyngeal specimens of known samples from high, medium, low, and very low viral load were mixed with SARS-CoV-2 negative nasopharyngeal specimens of known samples in 1:4 ratio, followed by analysis using Truenat. Furthermore, each sample in that pool was tested individually. Pooled sample testing was also done on the samples of unknown status.

Results: The results of the present study showed cycle threshold (Ct) values of pooled sample with SARS-CoV-2 positive RNA of high, medium, low, and very low viral load were 16.8, 24.22, 28.2, and 33.43, compared to Ct values of individual samples of 16.43, 22.0, 28.00, and 33.00, respectively.

Conclusion: These results suggest that the Ct values of pooled samples were in agreement with Ct values of individual samples indicating the validity of pooled sample testing for screening SARS-CoV-2 using Truenat.

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The following core competencies are addressed in this article: Medical knowledge, Patient care and procedural skills systems-based practice, Practice-based learning and improvement.

Keywords: Corona virus disease 2019, cycle threshold value, real-time polymerase chain reaction, sample pooling, severe acute respiratory syndrome corona virus-2, Truenat™ Beta CoV

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INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) causing coronavirus disease 2019 (COVID-19) has been creating havoc across the world. The viral disease was first identified in Wuhan city of Hubei province in China on December 12, 2019 and since then globally 298,915,721, laboratory confirmed cases of COVID-19, including 5,469,303 deaths were reported as of January 9, 2022.[1,2] SARS-CoV-2 has been continuously emerging into new variants through mutations in the spike gene of the SARS-CoV-2. The mutations may lead to increased transmissibility, high infectivity, and less responsive to treatments for COVID-19. Alpha, beta, and delta SARS-CoV-2 are few such SARS-CoV-2 variants, which have been associated with the new waves of infection in the recent past.[3] A new SARS-CoV-2 variant named Omicron variant (B.1.1.529 variant) with more than 30 mutations and with high transmissibility rate has been identified by the World Health Organization (WHO) in November 2021.[4]

To date, no specific, definitive approved antiviral therapeutic drugs with proven clinical efficiency available against SARS-CoV-2 infection and the only way is to contain the disease by following nonpharmaceutical interventions or mitigation measures as suggested by WHO.[5] The effective mitigation involves rapid laboratory based diagnostic testing to identify the COVID-19 infection, and hence, the WHO Director-General Ghebreyesus advised all countries to test, test, test as a best way to contain the pandemic.[6]

Studies conducted on the occurrence of asymptomatic COVID-19 cases suggest that around 10%–30% of COVID-19 cases reported were asymptomatic.[7-9] Hence, rapid and accurate identification of presymptomatic and asymptomatic cases is very crucial in the effective control of silent spread COVID-19. Systematic testing of the suspected individuals using molecular diagnostic tools is important for the identification of COVID-19 patients and tracing COVID-19 patient close contacts to curb the spread of the virus. However, a major hindrance of containing COVID-19 in several developing nations is the lack of large scale diagnostic testing and done only on symptomatic patients due to resource constraints such as poor laboratory capabilities, high cost and in some places overwhelmed on testing laboratories.

Truenat™ Beta CoV is a chip-based real-time polymerase chain reaction (PCR) test for the detection of coronavirus RNA in human nasopharyngeal and oropharyngeal specimens.[10] TrueNat is an indigenous diagnostic test originally designed (Truenat *Mycobacterium tuberculosis* [MTB]™) for the detection of drug-resistant MTB isolates in sputum specimens.[11] Later, the TrueNat system has been validated by the India's apex biomedical research body, i.e., *Indian Council of Medical Research* (ICMR), New Delhi and approved it as point of care test for the first line screening test for diagnosis of COVID-19 in April, 2020. Truenat Beta CoV test is stand on the detection of the E gene, which is commonly present in SARS-CoV causes SARS and SARS-CoV-2 causes COVID-19. Hence, individuals tested positive with Truenat Beta CoV (E gene screening assay) may be confirmed with S gene or RdRP gene or N gene reverse transcription (rRT)-PCR for the accurate identification of SARS-CoV-2.[12-14] TrueNat is an easy, quick, user friendly, and robust real-time PCR-based diagnosis technique which can be used in even at resource-limited settings.[15-17]

Pooled sample testing involves mixing of specimens from multiple individuals in a single tube and screening through rRT-PCR. If the pool test results positive then pool de-convolution will be done i.e., rRT-PCR will be done for the individual samples of the positive pool. Pooled sample testing was first introduced in 1943 by Robert Dorfman to large scale screening of syphilis in US military men.[18] Since then, the Dorfman approach has been applied in screening infectious diseases such malaria,[19]

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influenza[20] and Chlamydia[21] and during early stages of HIV pandemic.[22] Pooled sample testing approach has been demonstrated successfully for screening of SARS-CoV-2 in Germany,[23] USA,[24,25] and Israel[26] India[27,28] and been implemented for large scale screening of COVID-19. ICMR also suggested testing laboratories to use sample pooling for molecular testing in areas with low (<2%) infection positivity and for surveillance or survey studies in areas with 2%–5% infection positivity from the existing data.[29] The overall success rate of pool or group testing depends upon the incidence of the infectious disease in the population, specificity, sensitivity, and detection limits of the diagnostic test employed.[24,30]

The number of COVID-19 tests conducted per million population in India is low compared with the figures reported for UK (29, 412), US (36,961), Russia (50,381), and Italy (51 347). There is an urgent for increasing the molecular confirmatory tests to identify the asymptomatic carriers of COVID-19 to curb further spread of disease.[31] In this context, the present study is aimed to assess the detection of SARS-CoV-2 RNA in pooled samples from multiple individuals using Chip-based Real Time PCR Test (Truenat™ Beta CoV) while maintaining the reliability of the test and conserving the resources.

MATERIALS AND METHODS

The prospective evaluation study was carried out at nodal COVID-19 testing laboratory of Damien Foundation TB research centre. The center is a TB culture and drug susceptibility testing (C and DST) referral laboratory accredited by the National Mycobacteriology Accreditation System of Central TB Division Ministry of Health, Govt. of India. Government of Andhra Pradesh approved and converted Damien TB centre into COVID-19 nodal testing laboratory. The study was carried out on the left over samples received at nodal testing laboratory, Nellore, Andhra Pradesh, India, under BSL2 laboratory facilities. Nasopharyngeal swabs received in Trueprep® Auto Transport medium swab specimen tube containing viral lysis media (VLM) at nodal testing laboratory were processed following the recommended protocols of ICMR, New Delhi, Govt. of India. Initially, evaluation was done using known samples from laboratory confirmed COVID-19 positive and negative patient samples received during April and May, 2020. The present evaluation study was considered exemption as it was taken up as a part of the COVID-19 testing study. Further the results of the study were not considered for declaring patient status. The authors declare that the study was found to be noninterventional, and exempt by the local Ethics Committee.

Nucleic acids extraction (RNA) extraction

Pooled sample size of 5 was used with a VLM sample volume of 100 µl from each sample to make the final volume of l. SARS-CoV-2 positive nasopharyngeal specimens of high, medium, low, and very low viral load (as specified by the Manufacturer) were mixed with SARS-CoV-2 negative nasopharyngeal specimens of known samples in 1:4 ratio (1 each positive nasopharyngeal specimens and 4 negative nasopharyngeal specimens) and isolation of RNA was done. 500 µL of individual sample VLM was also taken into the lysis buffer tube and extraction of RNA was done separately. RNA extraction was done using Trueprep AUTO Universal Cartridge based Sample Prep Kit and Trueprep AUTO Universal Cartridge ®based Sample Prep Device following the manufacturers' instructions (Molbio Diagnostics Pvt. Ltd. Goa, India).

RNA isolated from pooled samples and individual samples were run on Truelab™ Real Time micro PCR Analyzer following the manufacturer's instructions. Truenat™ Beta CoV/Truelab™ Real-Time micro PCR Analyzer works on the principle of real time rRT PCR based on Taqman chemistry. Briefly, 6 l of the purified RNA isolated from the pooled and individual samples were added to the microtube containing the freeze dried PCR components (Supplied by Manufacturer) and allowed to stand for 30–60 s to get a clear solution. 6 l of the clear solution of template and PCR reagents were loaded onto the reaction well of the Truenat™ Beta CoV chip. Output is read as cycle threshold (Ct), a number of amplification cycles needed to cross the background signal. A clear horizontal amplification curve occurs in case of negative samples as there is no PCR amplification. The amount of target nucleic acid sequence present in the test sample is inversely proportional to the Ct values. After the completion of test run, the results are displayed are as "Detected" or "Not Detected" in case of positive and negative samples, respectively. Truelab™ PCR Analyzer also gives the viral load as "HIGH," "MEDIUM," "LOW," or "VERY LOW" for the COVID-19 positive samples. The reliability of the Truenat™ Beta CoV was assessed from the amplification of internal positive control (IPC) (human RNase P gene) to

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know proper sample collection, RNA purification and rRT-PCR. The human RNase P gene is a full process internal control which goes through every step the test specimen undergoes – from RNA isolation to rRT-PCR step thereby corroborate the test from specimen to result. RNase P will co amplifies along with positive samples case and shift or absence Ct value of RNase P beyond a preset range in case of negative samples cancels the test run. The validity of the test run is also displayed based on amplification of RNase P and the test results can be acquired to the laptop/desktop via Wifi network or can be taken as printout using the Truelab™ micro PCR printer.[14]

The study analyzes the test results of individual and pooled sample data for any significant difference in testing methods using Mann–Whitney *U*-test. The test was conducted using SPSS 16.0 version (SPSS, Inc., Chicago, USA).

RESULTS

Optimal pool testing was initially evaluated using online Shiny App for pooled testing tool as explained at https://www.chrisbilder.com/shiny. **A**lthough accurate COVID-19 prevalence rate in Nellore District of Andhra Pradesh, India is not known, the observed disease prevalence rate from samples received the different locations of Nellore has been considered as 2%. Optimum pooling size suggested by ICMR was evaluated with the following assumptions and parameters were given as inputs for shiny tool for hierarchical two stage pooling algorithm, TrueNat testing specificity and sensitivity as 100%, COVID-19 prevalence rate as 2% and pool size of 5. The algorithm results indicated that the two-stage hierarchical testing reduces the expected number of tests by 70% with pool size of 5 when compared to individual testing (https://www.chrisbilder.com/shiny/).

In the present study, threshold cycle (Ct) values E-gene of SARS-CoV-2 was evaluated in pooled sample and unpooled (Original) samples of known and unknown cases for the suitability of the pooled sample testing. Based on literature and test results of the Shiny algoritham pool size of 5 was chosen for testing. Comparison of Ct values between the pooled samples mixed in the ratio of one positive nasopharyngeal swab with the four negative nasopharyngeal swabs and individual of the pool indicate that pool size of five is good pool size for the accurate detection of corona virus. Four different pools were taken with VLM from patients with high, medium, low and very low viral load in 1: 4 ratio i.e., one each E-positive sample and four E-gene negative samples. One control pool with all 5 from E-gene negative samples was used as control. Pooling of samples was done before RNA isolation and then RNA isolation, rRT-PCR was done on Truenat™ Beta CoV/Truelab™ Real Time micro PCR Analyzer. RNA also isolated from COVID-19 positive patients and rRT-PCR was done on Truenat™ Beta CoV/Truelab™ Real Time micro PCR Analyzer separately. The Ct values for E-gene ranges from 16.8 to 33.43.

The Ct values of pooled samples from positive sample from high, medium, low, and very low viral load samples were 16.8, 24.22, 28.2, and 33.43, respectively [Table 1 and Figures 1 and 2]. Similarly, the Ct values of original samples of the pool from high, medium, low, and very low viral load samples were 16.43, 22.00, 28.00, and 33.00, respectively [Table 1 and Figures 1 and 2]. The Ct values of E-gene in original positive specimens and pools were below 35 and considered as positive. The difference in Ct values between pooled and original individual positive samples (Ct_{Pool}-$Ct_{Original\ Positive\ Sample}$) shows that there is only minimal difference and the values high, medium, low, and very low viral load samples were 0.37, 2.22, 0.20, and 0.43, respectively [Table 1 and Figure 3]. The data collected are subjected to Mann–Whitney *U*-Test for determining the difference in threshold cycles between individual sample and pooled sample data. The test results reveal that there is insignificant difference in threshold cycle values among the individual and pooled sample data mean ranks (Mean Rank: Individual –5.00, Pooled −4.00, U = 6.00, P = 0.564). Hence, it is evident that there is no difference in the pooled sample testing and testing samples individually for COVID-19 [Table 2]. No E-gene amplification was observed in negative control

Table 1: Comparison of Ct values between the unspooled and pooled samples mixed in one positive nasopharyngeal swabs with the four negative nasopharyngeal swabs

Sample number	E gene		
	Ct value of pooled samples (1 positive: 4 negative)	Ct value of individual or original sample	Difference in Ct value
High viral load	16.8	16.43	0.37
Medium viral load	24.22	22.0	2.22
Low viral load	28.8	28.0	0.8
Very low viral load	33.43	33.0	0.43

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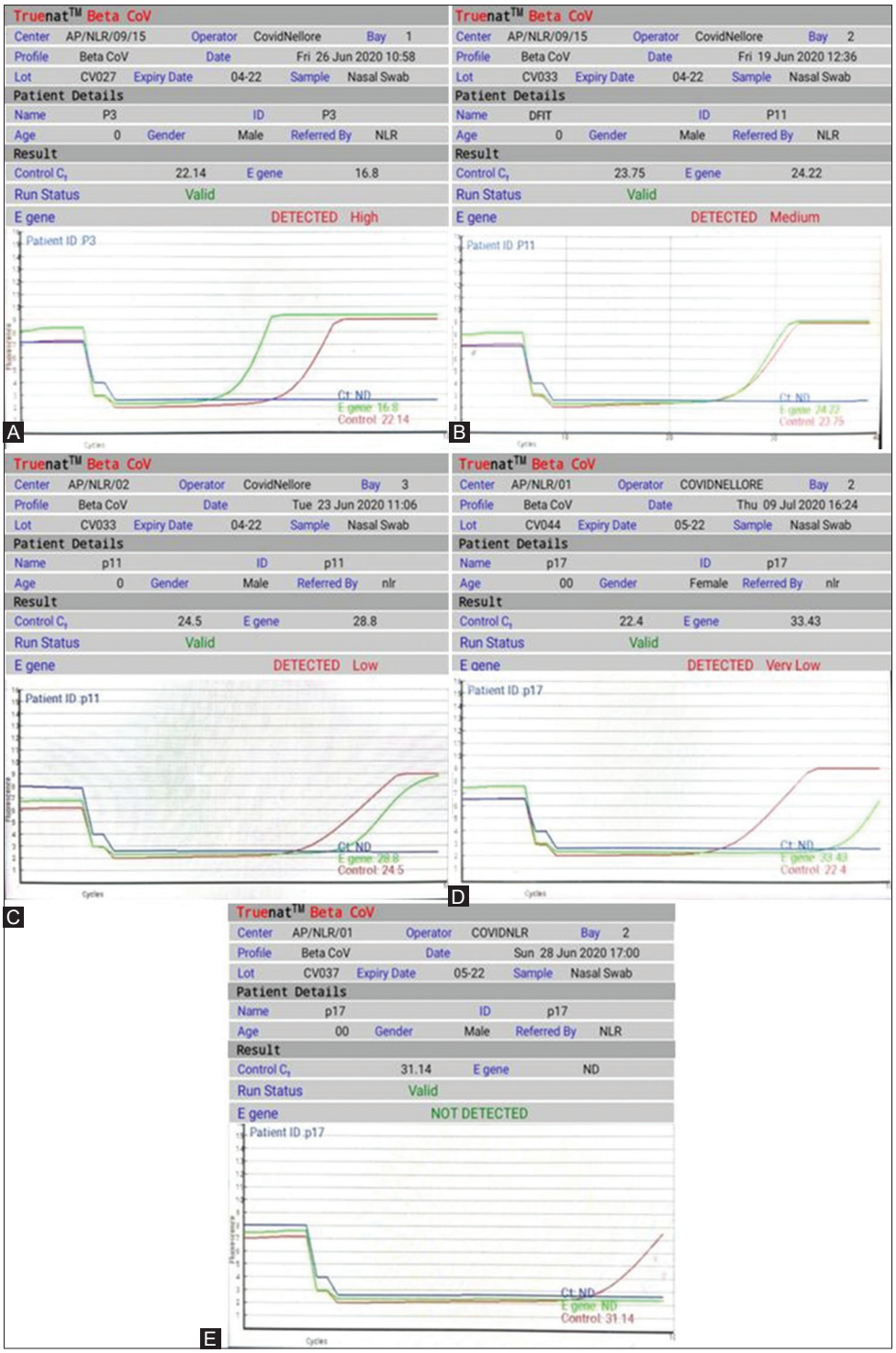

Figure 1: Screenshots of pooled sampling rRT-PCR test results formats as displayed on Real Time Quantitative micro Truelab™ PCR Analyzer. rRT-PCR: Real-time reverse transcription polymerase chain reaction (A: High Viral Load, B: Medium Viral Load, C; Low Viral Load, D: Very low Viral Load & E: negative Control)

Table 2: Mann–Whitney U-test analysis of pooled sample and individual sample testing

Group	Threshold cycles Ct – (mean rank)	Mann–Whitney U (statistic)	Asymptotic Significant (two-tailed)
Individual	5.00	6.00	0.564
Pooled	4.00		

pool with all 5 nasopharyngeal swabs from individuals with negative status. The pooled sample testing done with all 5 negative samples remain valid as there was clear amplification of IPC. No false-positive results were seen in our study. Subsequently, we applied

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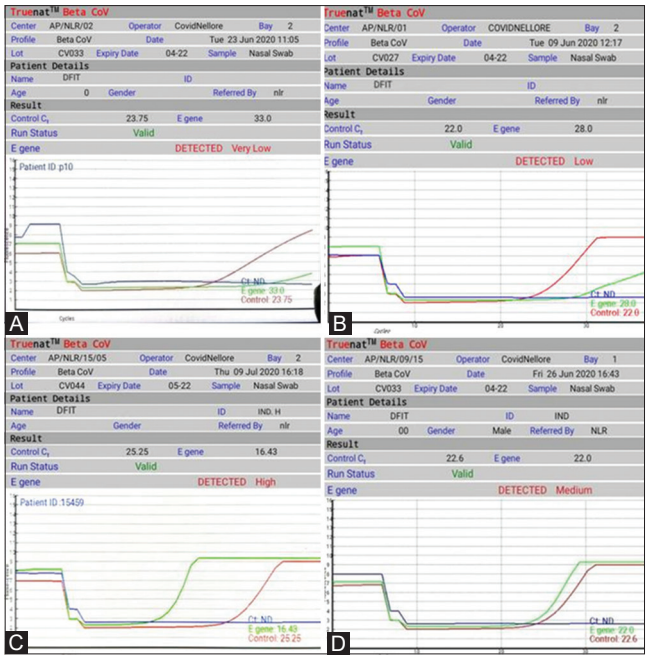

Figure 2: Screenshots of individual sample rRT-PCR test results formats as displayed on Real Time Quantitative micro Truelab™ PCR Analyzer. rRT-PCR: Real-time reverse transcription polymerase chain reaction. (A: High Viral Load, B: Medium Viral Load, C; Low Viral Load, D: Very low Viral Load)

pooled sample testing for the detection of E-gene in samples from patients with unknown status. Total 500 nasopharyngeal swabs collected during May 15 to June 25, 2020, were pooled in groups of 5 to create 100 pools. Then, the RNA isolation from the pools was done and rRT-PCR was done on TrueNat system. Five pools tested positive out of 100 pools, subsequent testing respective individual samples of positive pools detected 7 positive individuals with a prevalence rate of 1.4%. Overall, time taken for the pooled sample testing and identifying positive individual is only 4 h.

DISCUSSION

During the present COVID-19 pandemic, availability of RNA extraction kits and rRT-PCR testing kits has become a most important limiting factor in screening the large number of people to know the disease prevalence status and accurately determine the disease prevalence rate. In The sufficient COVID-19 testing laboratories and enough trained technical persons are lacking in majority of the states in India are not having sufficient number of. As a result enormous number of ($\sim 10^3$–10^4) samples to be tested are in waiting list in each nodal COVID-19 testing laboratory. Due to limited availability of testing, COVIID-19 diagnostic tests are done only to patients who have

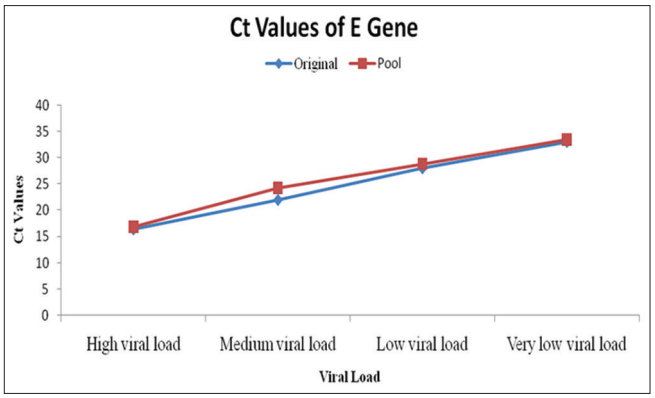

Figure 3: Graphical representation of Ct values between the original and pooled samples mixed in one positive Nasopharyngeal swabs with the four negative nasopharyngeal swabs. Ct: Cycle threshold

visible symptoms, travel history and to certain extent primary contacts. In addition, due to delay in testing, the chances of spread of COVID-19 might increase and makes situation the worst. Moreover, the recent studies indicate that 20%–30% COVID-19 cases are asymptomatic and a few are mild cases. Testing every individual who is in close vicinity of the COVID-19 patient, and all primary and secondary contacts would enable to reduce the rapid spread of COVID-19. Further, the time taken for testing the suspected cases and associated cost is also very high. RT-qPCR study conducted by Yelin *et al*., reported that the SARS-CoV-2 RNA of a single positive specimen can be detected even in pooled sample size up to 32, with an expected false negative rate of 1 out of 10 (10%). Pure RNA instead of original sample specimens was mixed in multi pool study carried out by Yelin *et al*.[26] Similarly, using Pooling-Based Efficient SARS-CoV-2 Testing, Shental *et al*., identified 1–5 SARS-Cov-2 positive carriers in 48 pools grouped from 384 samples providing an 8 fold increase in the COVID-19 testing efficiency.[9]

In this context, pooled sample testing method reduces the time for screening large of individuals, reagents required for testing and most importantly the cost. The average cost for testing one sample by TrueNat testing method is INR1700 (22.48$). Use of sample pooling (pool size of 5), the cost incurred for testing one individual sample is only INR 340 (4.5$) and pooled sample testing reduces the cost by 80%. The results of the present study suggest that COVID-19 screening using pooled sample testing method detects even if the viral load is very low as indicated by the TrueNat without increasing the additional number of cycles and with satisfactory diagnostic accuracy. We

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pooled sample prior to RNA extraction, doing so, bottlenecks associated with the RNA extraction will be reduced. As pool testing follows standard approved proved equipment, reagents and protocols, pooled testing method can be adapted in existing COVID-19 testing laboratories. Immediate implementation of pooled testing may increases the current screening capacity and thus increasing the testing of large number of individuals in the community and hence reducing the community transmission. Success of sample pooling depends upon quality and adequate sample collection, efficient extraction and sensitive detection methods. The pool sample testing has some limitations such as intricate work flow, availability of the skilled lab workers, collection and appropriate storage of samples. Further, sample pool testing can only be done when the prevalence of disease positivity is low in the community and hence, pool sampling can't be used during the peak of pandemic. Lastly occurrence of false negative results is high especially with low viral load samples or samples with borderline Ct values.

CONCLUSIONS

The findings of the present study suggest that the Ct values of pooled samples were in agreement with Ct values of individual samples indicating the validity of pooled sample testing for screening SARS-CoV-2 using Truenat. Further, a very low viral load of SARS-CoV-2 in multipool nasopharyngeal samples can be detected using Chip-based Real Time PCR Test (Truenat™ Beta CoV). Testing of pooled samples provides alternative approach to increase testing capacity.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Research quality and ethics statement

The authors of this manuscript declare that the applicable EQUATOR Network (https://www.equator-network.org/) reporting guidelines were followed. The authors also declare that the study was found to be exempt by the local Ethics Committee.

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