Indian J Med Res 152, July & August 2020, pp 88-94
DOI: 10.4103/ijmr.IJMR_2304_20

Quick Response Code:

Pooled testing for COVID-19 diagnosis by real-time RT-PCR: A multi-site comparative evaluation of 5- & 10-sample pooling

Ira Praharaj[1], Amita Jain[3], Mini Singh[4], Anukumar Balakrishnan[5], Rahul Dhodapkar[6], Biswajyoti Borkakoty[7], Munivenkatappa Ashok[8], Pradeep Das[9], Debasis Biswas[10], Usha Kalawat[11], Jyotirmayee Turuk[12], A.P. Sugunan[5], Shantanu Prakash[3], Anirudh K. Singh[10], Rajamani Barathidasan[6], Subhra Subhadra[12], Jyotsnamayee Sabat[12], M.J. Manjunath[8], Poonam Kanta[4], Nagaraja Mudhigeti[11], Rahul Hazarika[7], Hricha Mishra[3], Kumar Abhishek[9], C. Santhalembi[5], Manas Ranjan Dikhit[9], Neetu Vijay[2], Jitendra Narayan[2], Harmanmeet Kaur[2], Sidhartha Giri[1] & Nivedita Gupta[1]

[1]Divsion of Epidemiology & Communicable Diseases, Indian Council of Medical Research, [2]Department of Health Research, Ministry of Health & Family Welfare, New Delhi, [3]Department of Microbiology, King George's Medical University, Lucknow, Uttar Pradesh, [4]Department of Virology, Postgraduate Institute of Medical Education & Research, Chandigarh, [5]ICMR-National Institute of Virology, Kerala Unit, Alappuzha, Kerala, [6]Department of Microbiology, Jawaharlal Institute of Postgraduate Medical Education & Research, Puducherry, [7]Regional Medical Research Centre, Dibrugarh, Assam, [8]ICMR-National Institute of Virology, Bangalore Unit, Bengaluru, Karnataka, [9]ICMR-Rajendra Memorial Research Institute of Medical Sciences, Patna, Bihar, [10]Department of Microbiology, All India Institute of Medical Sciences, Bhopal, Madhya Pradesh, [11]Department of Microbiology, Sri Venkateswara Institute of Medical Sciences, Tirupati, Andhra Pradesh & [12]ICMR-Regional Medical Research Centre, Bhubaneswar, Odisha, India

Received May 31, 2020

***Background & objectives*: Public health and diagnostic laboratories are facing huge sample loads for COVID-19 diagnosis by real-time reverse transcription-polymerase chain reaction (RT-PCR). High sensitivity of optimized real-time RT-PCR assays makes pooled testing a potentially efficient strategy for resource utilization when positivity rates for particular regions or groups of individuals are low. We report here a comparative analysis of pooled testing for 5- and 10-sample pools by real-time RT-PCR across 10 COVID-19 testing laboratories in India.**

***Methods*: Ten virus research and diagnostic laboratories (VRDLs) testing for COVID-19 by real-time RT-PCR participated in this evaluation. At each laboratory, 100 nasopharyngeal swab samples including 10 positive samples were used to create 5- and 10-sample pools with one positive sample in each pool. RNA extraction and real-time RT-PCR for SARS-CoV-2-specific *E* gene target were performed for individual positive samples as well as pooled samples. Concordance between individual sample testing and testing in the 5- or 10-sample pools was calculated, and the variation across sites and by sample cycle threshold (C_t) values was analyzed.**

***Results*: A total of 110 each of 5- and 10-sample pools were evaluated. Concordance between the 5-sample pool and individual sample testing was 100 per cent in the C_t value ≤30 cycles and 95.5 per cent for C_t**

values ≤33 cycles. Overall concordance between the 5-sample pooled and individual sample testing was 88 per cent while that between 10-sample pool and individual sample testing was 66 per cent. Although the concordance rates for both the 5- and 10-sample pooled testing varied across laboratories, yet for samples with C_t values ≤33 cycles, the concordance was ≥90 per cent across all laboratories for the 5-sample pools.

***Interpretation & conclusions*: Results from this multi-site assessment suggest that pooling five samples for SARS-CoV-2 detection by real-time RT-PCR may be an acceptable strategy without much loss of sensitivity even for low viral loads, while with 10-sample pools, there may be considerably higher numbers of false negatives. However, testing laboratories should perform validations with the specific RNA extraction and RT-PCR kits in use at their centres before initiating pooled testing.**

Key words Concordance - COVID-19 - *E* gene - pooling - real-time RT-PCR - SARS-CoV-2 - sensitivity

Extensive testing for SARS-CoV-2 is one of the most important components of COVID-19 control strategy at present[1]. Timely and accurate reporting can lead to proper contact tracing and effective containment measures. Real-time reverse transcription-polymerase chain reaction (RT-PCR)-based molecular assays for various SARS-CoV-2 gene targets are the mainstay of diagnosis for COVID-19 at present[2,3]. Given the evolving strategies for testing worldwide which now also include testing asymptomatic individuals with pertinent contact history, the load of sample testing has grown manifold in laboratories testing for SARS-CoV-2. In this scenario, many countries face shortage of resources and testing kits for detection of SARS-CoV-2. This situation is especially pronounced for low- and middle-income country settings. Shortage in critical reagents required for real-time RT-PCR has led to the emphasis on various strategies for efficient use of resources required for testing. Among the different strategies, pooling of respiratory samples, specifically nasopharyngeal samples, has received attention, given the simplicity of the approach and the fact that there are no additional requirements in terms of equipment or reagents for this approach.

During a pandemic, a high load of samples requires to be tested, especially for containment measures, but the positivity rates are low or show a wide variation from place to place. In such a scenario, pooling of samples can be considered a viable option to conserve resources and time for testing a large number of samples. While pooling of respiratory samples for the detection of RNA viruses such as influenza viruses has been evaluated[4], it has not been widely used for diagnostic sample testing.

With the high sensitivity of real-time RT-PCR-based tests and the low prevalence of COVID-19 infection in many areas and regions of India, pooled testing of respiratory samples has the potential of increasing the testing capacity considerably. Mathematical models and epidemiological projections have suggested different pooling sizes, which might be feasible and effective for handling testing needs in regions with different positivity rates[5]. While deciding on the appropriate pool size, biological and experimental feasibility is very important and should be evaluated. It is necessary to strike a balance between the resources that are saved because of pooled sample testing and the potential loss of sensitivity of the real-time RT-PCR assay that such testing might entail.

The objective of the present study was to do a comparative analysis of pooled testing using 5- and 10-sample pools by real-time RT-PCR performed in 10 different COVID-19 testing laboratories across India.

Material & Methods

All the 10 laboratories involved in this evaluation were virus research and diagnostic laboratories (VRDLs) supported under the Department of Health Research-Indian Council of Medical Research (DHR-ICMR) scheme[6] and were designated real-time RT-PCR testing laboratories for COVID-19. These 10 laboratories were: ICMR-National Institute of Virology (ICMR-NIV), Bangalore Unit, Bengaluru; Jawaharlal Institute of Postgraduate Medical Education & Research (JIPMER), Puducherry; King George's Medical University (KGMU), Lucknow; ICMR-NIV, Kerala Unit, Alappuzha; Postgraduate Institute of Medical Education & Research (PGIMER), Chandigarh; All India Institute of Medical Sciences (AIIMS), Bhopal; ICMR-Regional Medical Research Centre (ICMR-RMRC), Bhubaneswar; ICMR-RMRC, Dibrugarh; ICMR-Rajendra Memorial Research Institute of

Medical Sciences (ICMR-RMRIMS), Patna; and Sri Venkateswara Institute of Medical Sciences (SVIMS), Tirupati. These described experiments were carried out during May 8-20, 2020, using samples already tested for COVID-19 diagnosis as part of the national COVID-19 strategy.

Nasopharyngeal swab samples in the viral transport medium (VTM) previously tested positive and negative for SARS-CoV-2, and appropriately stored at −80°C, were used. For the set of experiments described below, all the samples used were anonymized and used for evaluating a pooling process for increasing testing capacity and efficiency. No age, gender or any other personal identifiers corresponding to samples were accessed or used during testing or analysis. A standard template for pooling of samples was shared with each of the participating laboratories. A volume of 200 µl of VTM from each of the individual samples was used for pooling. Each pool of five samples included one positive and four negatives. Each pool of 10 samples included one positive and nine negatives. Therefore, the total volume of a 5-sample pool was 1 ml and that of a 10-sample pool was 2 ml. The participating laboratories were instructed to include positive samples with a range of cycle threshold (C_t) values, with a majority in the range of C_t value 30 and above. In addition, each laboratory also included one 5-sample pool and one 10-sample pool comprising negative tested samples.

RNA extraction from individual and pooled samples: RNA was extracted from both 5-sample and 10-sample pools, as well as the individual samples using the same RNA extraction kit. A volume of 200 µl of the pooled sample was used for RNA extraction. Participating laboratories were instructed to use the same extraction kits for individual samples, 5-sample pools, as well as 10-sample pools. The individual samples, as well as the pools, were included in the same extraction batch, and the same aliquot of sample was used for individual sample testing as well as creating 5- and 10-sample pools for RNA extraction. Each laboratory also included two negative pools of samples, one with five negative samples and another with 10 negative samples.

RNA extraction kits used by the different laboratories included QiaAmp Mini Viral RNA Kit (Qiagen, Germany) used by six laboratories, MGIEasy Nucleic Acid Extraction Kit (MGI Tech Co., Ltd., China) by two laboratories and HiPura Viral RNA Extraction Kit (HiMedia, Mumbai) used by a single laboratory.

Real-time RT-PCR for SARS-CoV E gene: Single-step real-time RT-PCR for SARS-CoV-2 targeting the *E* gene was performed on the extracted RNA from individual samples as well as the sample pools. Participating laboratories used the same RT-PCR kit for *E* gene detection as was being used on a regular basis for COVID-19 diagnosis at the centre. Six of the laboratories used the *E* gene screening assay as per the protocol described earlier[6]. Two laboratories used the TIB Molbiol 2019 nCoV Kit (TIB Molbiol, Germany). One laboratory each used Standard M nCoV Real-Time Detection Kit (SD Biosensor Inc., Republic of Korea) and PathoDetect COVID-19 Detection Kit (Mylab Discovery Solutions, Maharashtra). RNA extracted from individual samples as well as the 5- and 10-sample pools were included in the same real-time RT-PCR assay batch to avoid inter-assay variation. Concordance between individual sample testing and pooled sample testing was calculated and expressed in percentages.

Results

A total of 110 5-sample pools and an equal number of 10-sample pools were evaluated across the 10 laboratories. This included a total of 1000 nasopharyngeal/oropharyngeal swab samples which had already been tested for SARS-CoV-2 using established methods[2]. Of these, 100 samples were confirmed positive for SARS-CoV-2 and were included for the individual sample as well as 5- and 10-sample pools testing.

Positive samples included in creating pools ranged from a C_t value of 23 cycles to a maximum of 35.9 cycles. For a majority of the 5- and 10-sample pools (77 of the 110), the positive samples included in the positive pools had C_t values more than 30 cycles (Figure).

Concordance between individual sample testing and the pooled testing with five and 10 samples was calculated. With 5-sample pooling, the overall concordance was calculated to be 88 per cent. In 10-sample pools, positive samples could be detected in only 66 of 100 (66%) of pools evaluated. The concordance varied considerably with the viral load (Table I). For positive samples with C_t values less than or equal to 30 cycles, the concordance rates with

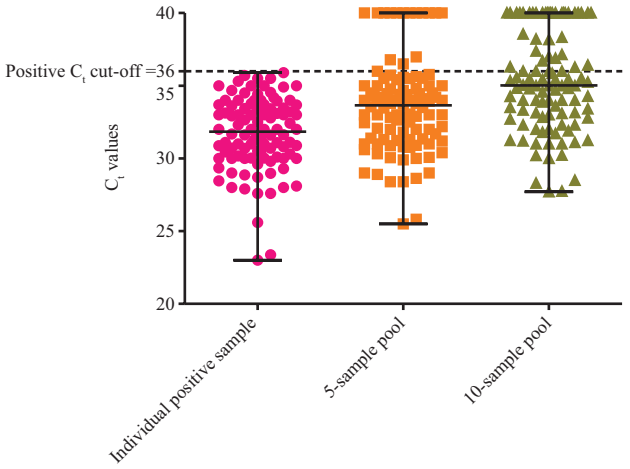

Figure. C_t value distribution for individual sample testing compared to 5-sample and 10-sample pooled testing. Note: For samples where no amplification was seen in the real-time RT-PCR assay, a C_t value of 40 cycles was assigned and used for plotting purposes. RT-PCR, reverse transcription-polymerase chain reaction.

pooled sample testing were high (100% for 5-sample pools and 95.6% for 10-sample pools).

Concordance rates also varied across testing laboratories. For 5-sample pools, concordance rate varied from a minimum of 70 to 100 per cent (Table II). Four of the 10 laboratories had a 100 per cent concordance between the 5-sample pools and the individual positive samples. The concordance rates between 5-sample pools and individual sample testing for samples with C_t values less than or equal to 33 cycles were 90 per cent or more across all testing laboratories. For the 10-sample pools, the concordance rate varied from a minimum of 50 per cent to a maximum of 90 per cent across laboratories (Table II).

On an average, C_t values obtained with the 5-sample pooled testing exceeded individual sample testing by 2.18±1.86 cycles while C_t values obtained with the 10-sample pooling exceeded individual sample testing by 3.81±2.26 cycles. In a real-time RT-PCR assay with 100 per cent efficiency, a difference of approximately 3.3 cycles was expected between samples with a 10-fold difference in viral load. The average C_t value differences between individual and pooled sample testing were consistent with this.

Discussion

Pooled sample testing has been considered as a simple and practical approach for improving the testing output while minimizing the resources being utilized for real-time RT-PCR[7]. Several studies from different countries have reported different pooling strategies[7-13] and are included in Table III.

Based on the comparative analysis of the 5- and 10-sample pooling in the present study, 5-sample pooling was better than 10-sample pooling as the concordance rates with individual sample testing were high and false-negativity rates were low. This was especially true for samples with C_t values less than 33 cycles. Concordance rates for samples with C_t values greater than 33 cycles were considerably less. With 10-sample pools, the concordance rates with individual sample testing were low and false-negativity rates were high at C_t values more than 30 cycles. Recent reports from the USA, Spain and Chile had similar results with the 5-sample pooling strategy[9,10,13].

While 5-sample pooling gave consistently better results compared to 10-sample pooling across the all testing sites, there was variation in the false-negativity rates. Though this could have been a function of the different RNA extraction and RT-PCR kits used across the different sites, it will be difficult to make any conclusions with the available data. It is recommended that before initiating pooled sample testing, laboratories perform validation experiments with the RNA extraction and RT-PCR kits being used. Further, the practical efficiency of sample pooling is a function

Table I. Concordance between pooled and individual sample testing

C_t value range for individual positive sample	Number of included positive samples	Concordance between pooled testing and individual testing	
		5-sample pooled testing versus individual testing, n (%)	10-sample pooled testing versus individual testing, n (%)
≤30 cycles	23	23/23 (100)	22/23 (95.6)
>30 and ≤33 cycles	44	42/44 (95.5)	35/44 (79.5)
>33 and <36 cycles	33	23/33 (69.7)	9/33 (27.3)
Overall	100	88/100 (88)	66/100 (66)

Table II. Laboratory-wise concordance between positive samples included for evaluation and detection in 5- and 10-sample pools

Name of the COVID-19 testing laboratory	RNA extraction kit used	RT-PCR kit used	Concordance between individual testing and 5-sample pool, n (%)	Concordance between individual testing and 10-sample pool, n (%)
ICMR-NIV, Bangalore Unit, Bengaluru	QIAamp Viral RNA Mini Kit (Qiagen, Germany)	*E* gene screening assay-NIV protocol*	90	80
JIPMER, Puducherry	MGIEasy Nucleic Acid Extraction Kit (MGI Tech Co., Ltd., China)	Standard M nCoV Real-Time Detection Kit (SD Biosensor, Republic of Korea)	70	50
KGMU, Lucknow	PureLink Viral RNA/ DNA Mini Kit (Invitrogen/Thermo Fisher Scientific, USA)	*E* gene screening assay-NIV protocol*	100	90
ICMR-NIV, Kerala Unit, Alappuzha	QIAamp Viral RNA Mini Kit (Qiagen, Germany)	*E* gene screening assay-NIV protocol*	80	70
PGIMER, Chandigarh	QIAamp Viral RNA Mini Kit (Qiagen, Germany)	TIB Molbiol 2019 nCoV Kit (TIB Molbiol, Germany)	100	80
AIIMS, Bhopal	MGIEasy Nucleic Acid Extraction Kit (MGI Tech Co., Ltd., China)	*E* gene screening assay-NIV protocol*	90	60
ICMR-RMRC, Bhubaneswar	QIAamp Viral RNA Mini Kit (Qiagen, Germany)	*E* gene screening assay-NIV protocol*	70	50
ICMR-RMRC, Dibrugarh	QIAamp Viral RNA Mini Kit, (Qiagen, Germany)	TIB Molbiol 2019 nCoV Kit (TIB Molbiol, Germany)	100	70
ICMR-RMRIMS, Patna	QIAamp Viral RNA Mini Kit (Qiagen, Germany)	*E* gene screening assay-NIV protocol*	100	70
SVIMS, Tirupati	HiPura Viral RNA Extraction Kit (HiMedia, Mumbai)	PathoDetect COVID-19 Detection Kit (Mylab Discovery Solutions, Maharashtra)	80	70

RT-PCR, reverse transcription-polymerase chain reaction; NIV, National Institute of Virology; JIPMER, Jawaharlal Institute of Postgraduate Medical Education & Research; KGMU, King George's Medical University; PGIMER, Postgraduate Institute of Medical Education & Research; AIIMS, All India Institute of Medical Sciences; RMRC, Regional Medical Research Centre; RMRIMS, Rajendra Memorial Research Institute of Medical Sciences; SVIMS, Sri Venkateswara Institute of Medical Sciences
Source: Ref. 6

Table III. Strategy for pooled real-time RT-PCR testing reported from different countries

Country	Pooling strategy	Size of pool	References
Israel	Pooling of extracted RNA	Pooling of extracted RNA (32/pool)	8
Chile	Pooling of nasopharyngeal samples in universal transport medium	5 samples/pool	9
Germany	Pooling of extracted RNA before RT-PCR amplification	Range of pool sizes of extracted RNA (4-30/pool)	7
Germany	Pooling of swabs directly in a 'pool container' after being placed in an 'archive' container	5 samples/pool	11
Israel	Combinatorial pooling strategy where each sample is a part of multiple pools. Liquid dispensing robot used to create pools.	348 patient samples were tested in 48 pools	12
USA	Pools of 5 samples 50 µl each with one positive in each pool were evaluated	5 samples/pool	10
Spain	Pooling of nasopharyngeal specimens from universal transport medium	Pool sizes of 5 samples/pool and 10 samples/pool evaluated	13

of the positivity rate for a particular country, State or region or even in the specific group of individuals being tested. Therefore, batch sizes for pooled testing or even the decision to follow pooled testing should be taken at the laboratory or regional levels, taking into consideration the positivity rates, the specific groups and categories being tested. Individual groups for whom there is a higher pre-test probability and those with serious manifestations should not be included in pooled testing but should be tested individually. Mathematical models have suggested that sample pooling strategy will work best in settings with low prevalence and individual subgroups with low clinical suspicion such as asymptomatic individuals[14].

In this analysis, pooling of nasopharyngeal swab samples in VTM was done before RNA extraction. Some recent studies and pre-prints have reported successful pooling of extracted RNA for up to 32 different samples[7,8]. Such an approach of performing RNA extraction and then pooling only saves resources at the RT-PCR step and not at the RNA extraction step. The RNA extraction step remains one of the most rate-limiting steps for SARS-CoV-2 RT-PCR[15] in terms of usage of reagents as well as time required. Besides, for the RNA extraction quality control step, separate runs for RNase P or other internal control need to be performed for each extracted RNA, and this nullifies to some extent the advantages of the pooling of RNA extracts.

The major limitation of our study was that not all the 10 participating laboratories used the same RNA extraction kit or RT-PCR kit for this exercise. While using the same RNA extraction kits as well as RT-PCR kits across the 10 different participating laboratories would have made this evaluation more rigorous, the decision to allow each laboratory to proceed with the current kits they were using for diagnostic testing for COVID-19 was based on the fact that given the current constraints in the availability of testing kits, in many instances, laboratories may not be able to use their preferred kits at this point of time.

In conclusion, the results from this multi-site comparison of the 5- and 10-sample pooling suggest that pooling five samples for SARS-CoV-2 detection by real-time RT-PCR may be an acceptable strategy without much loss of sensitivity for low viral loads. However, the results obtained highlight the need for each laboratory to perform validation runs at their centres and when using different RNA extraction and RT-PCR kits. It is also emphasized that pooling sizes will differ by the populations and group of individuals being tested as well as the positivity rates. These will continue to evolve during a pandemic, and therefore, guidelines for pooling samples must be revisited from time to time to ensure that they continue to be relevant and useful in a given context.

***Acknowledgment*:** Authors thank Shri Akash Dey from Clinton Health Access Initiative, New Delhi, for helpful discussions.

***Financial support & sponsorship*:** All the VRDLs participating in this evaluation were funded through the Department of Health Research scheme "Setting up a network of nation-wide network of laboratories for managing epidemics and national calamities".

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

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For correspondence: Dr Nivedita Gupta, Division of Epidemiology & Communicable Diseases, Indian Council of Medical Research, Ansari Nagar, New Delhi 110 029, India
e-mail: guptanivedita.hq@icmr.gov.in