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Brief Communication

Analysis of Multi-Sample Pools in the Detection of SARS-CoV-2 RNA for Mass Screening: An Indian Perspective

Sangeeta Deka, Deepjyoti Kalita, Amit Mangla, Ravi Shankar

Department of Microbiology, All India Institute of Medical Sciences, Rishikesh, Uttarakhand, India

Abstract

In the current COVID-19 crisis, many national healthcare systems are confronted with a huge demand for mass testing and an acute shortage of diagnostic resources. Considering group testing as a viable solution, this pilot study was carried out to find the maximum number of samples that can be pooled together to accurately detect one positive sample carrying the severe acute respiratory syndrome-coronavirus 2 viral RNA from different pools. We made different pool sizes ranging from 5 to 30 samples. Three positive samples, covering the common range of polymerase chain reaction (PCR) threshold cycle values (an indirect indicator of viral load) observed in our patients, were selected, and different pools were made with known negative samples. The pools underwent real-time qualitative PCR for the determination of effective maximum pool size. It was observed that up to 20-sample pools of all positive samples could accurately be detected in terms of both E gene and RdRp gene, leading to considerable conservation of resources, time and workforce. However, while deciding the optimal pool size, the infection level in that particular geographical area and sensitivity of the test assay used (limit of detection) have to be taken into account.

Keywords: COVID-19, group testing, pooling, reverse-transcriptase-polymerase chain reaction, SARS-CoV-2

Introduction

The ongoing COVID-19 pandemic is growing exponentially and has adversely affected the economy of many countries. With the total number of affected people worldwide crossing eight million, the total cases in India stood at 372,685 (18 June 2020).[1] It has become a major challenge for many countries to meet the increasing demand for diagnostic resources. Large-scale testing, early detection (including the asymptomatic silent-spreaders) and isolation of infected people are among the most effective strategies to control the spread of COVID.[2] The only reliable diagnostic tool for diagnosis, till date i.e., real-time reverse-transcriptase polymerase chain reaction (RT-PCR) is a labour-intensive technical procedure and relatively expensive compared to other screening methods commonly employed for viral diseases. Both antibody and antigen-based techniques, suffer from sensitivity and/or specificity issues. The latter method is advocated by the Government of India, in certain specific situations, given its reliable specificity (despite poor sensitivity).[3] Overall, group testing strategy could be a promising option for mass testing.

This study aims to find the maximum number of samples that can be pooled together to accurately detect one positive sample carrying the severe acute respiratory syndrome-corona virus 2 (SARS-CoV-2) RNA, to substantially reduce the number of tests, thereby conserving resources.

Materials and Methods

This exploratory study was conducted in the virology laboratory of our institute between 6 and 15 May 2020. Institutional review board/institute's ethics committee approval was obtained with approval number AIIMS/IEC/20/523 dated 08/08/2020. Three known positive samples covering commonly encountered range of cycle threshold (Ct) values (for E-gene 20.69, 25.30 and 29.72) and 99 known negative samples were selected.

Address for correspondence: Dr. Deepjyoti Kalita, Department of Microbiology, All India Institute of Medical Sciences, Level 4, Virbhadra Road, Rishikesh - 249 203, Uttarakhand, India.
E-mail: dkalita@gmail.com

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Table 1: Cycle threshold value of the three positive samples in different group (sample-pool) size [also refer to Figures 2 and 3]

	First positive		Second positive		Third positive	
	E-gene	RdRp-gene	E-gene	RdRp-gene	E-gene	RdRp-gene
Individual testing						
Ct (I)	20.69	20.33	25.30	26.32	29.72	30.02
Group size of 5						
Ct (5)	19.92	20.44	28.5	29.62	31.55	32.14
Ct difference	−0.77	0.11	3.20	3.30	1.83	2.12
Group size of 10						
Ct (10)	22.31	21.46	29.81	30.62	32.31	33.38
Ct difference	1.64	1.13	4.51	4.30	2.59	3.36
Group size of 15						
Ct (15)	23.29	22.46	31.11	32.04	33.13	33.79
Ct difference	2.60	2.13	5.81	5.72	3.41	3.77
Group size of 20						
Ct (20)	24.08	23.34	31.76	32.77	34.20	34.34
Ct difference	3.39	3.01	6.46	6.45	4.48	4.32
Group size of 25						
Ct (25)	25.09	24.33	32.34	33.15	35.72	-
Ct difference	4.40	4.00	7.04	6.83	6.00	
Group size of 30						
Ct (30)	25.94	25.15	33.62	33.77	-	-
Ct difference	5.25	4.82	8.32	7.45		

Ct: Cycle threshold

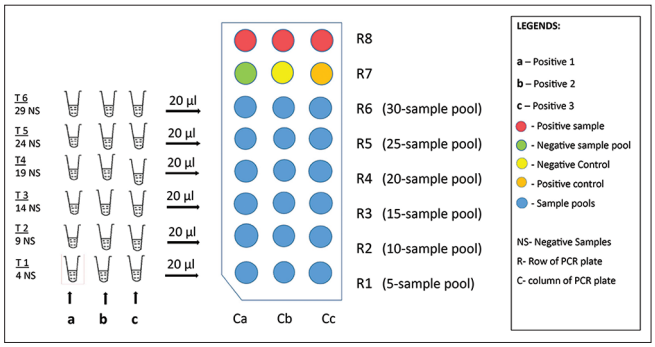

Figure 1: Arrangement of pools

Viral nucleic acid (RNA) was extracted using automated magnetic bead method (Thermo Scientific's KingFisher™ Flex duo system with the MagMAX™ Kit, 81 Wyman street Walttham, MA02451, USA) inside Bio-safety Cabinet Class II a. Extracted viral RNA elute (known negative and known positive) was used as samples in all further experiments.

Six series of tubes (T1 to T6), each series containing three tubes, were arranged [Figure 1] where all tubes in T1 were poured with 10 µl each of four unique negative samples (making 40 µl in each tube). Subsequently, T2 tubes were poured with nine unique negative samples each. Similarly, T3, T4, T5 and T6 series tubes received 14, 19, 25 and 29 numbers of unique COVID-19 negative samples, respectively. In the next phase, three known COVID-19-positive samples (marked as a, b and c, respectively) were taken, and a volume

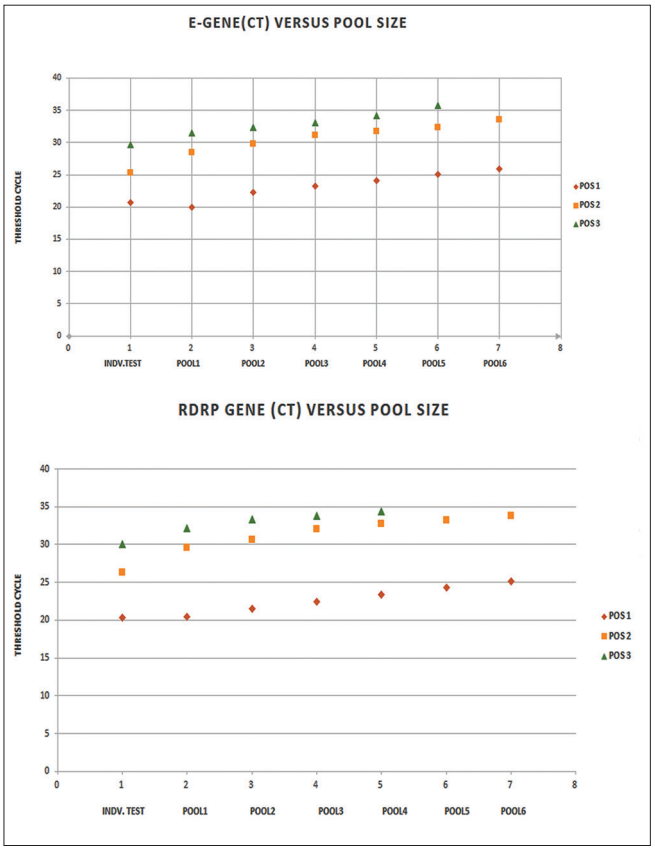

Figure 2: Relation of cycle threshold values for E-gene and RdRp gene versus pool size

of 10 µl of 'a' was poured into the first line of tubes of all the six (T1 to T6) series [Figure 1]. Similarly, 10 µl each from 'b' went to all the 2nd line of tubes and 10 µl from 'c' went to all tubes in the last line. This process yielded pools of 5, 10, 15, 20, 25 and 30 samples in T1, T2, T3, T4, T5 and T6 series tubes, respectively. Subsequently, 20-µl pooled samples, from each tube, were transferred to the corresponding wells of a PCR plate [Figure 1]. In the 7th row, the 1st well contained a pool of unique five negative samples, whereas the 2nd and 3rd wells were meant for PCR controls (positive and negative). Eight rows contained three positive samples for individual testing [Figure 1].

For SARS-CoV-2 RT-PCR testing, we used STANDARD-M nCoV Real-Time Detection kit (SD BIOSENSOR, Inc., Republic of Korea, Suwon-si, Gyeonggi-do, 16690, Republic Of Korea) on these 18 pools and the rest 6 wells targeting

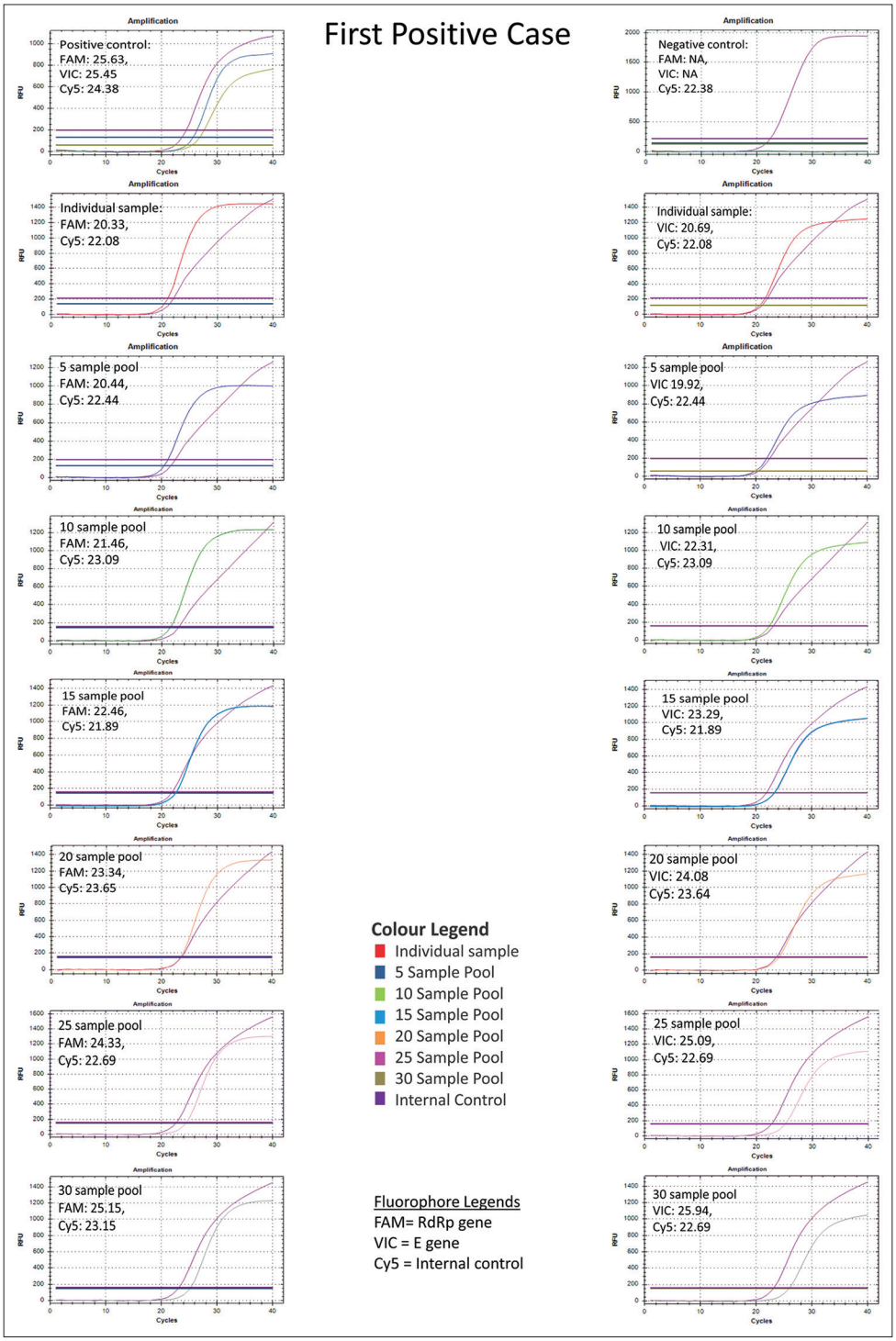

Figure 3: Amplification curves of various pools in the first positive case

E-gene and RdRp gene (besides manufacturer-provided Internal control [IC] target), in a Biorad CFX 96 Real time Thermocycler (Foster City, California, 94404, USA). FAM, VIC and CY5 channels were used for the detection of E-gene, RdRp gene and IC, respectively. Protocol was as per kit insert with Cut Ct <36. The kit's diagnostic sensitivity was 95% with limit of detection) 250 copy/ml for upper respiratory samples.[4]

Results

As depicted in Table 1, all the three positive samples could be reliably detected up to pool size 20 (for RdRp gene) and pool size 25 (for E gene). The first two positive samples were detectable up to pool size 30 for both the genes, however for the third positive sample, RdRp gene went undetectable in higher pool sizes [Table 1 and Figure 2]. The observed linearity in Figure 2 indicates that in most cases, there was no RNA interference with the reverse transcriptase or DNA polymerase enzyme. The pool of negative samples was negative for both E and RdRp genes. Ct value differences between pooled tests and individual positive samples (Ct_{pool}–$Ct_{positive}$ sample) were in the range of −0.77–8.32. Figures 3-5 depict the amplification of various targets (with IC s) in individual as well as selected group sizes. The positive control run provided Ct values for RdRp, e-gene and IC as 25.63, 25.45

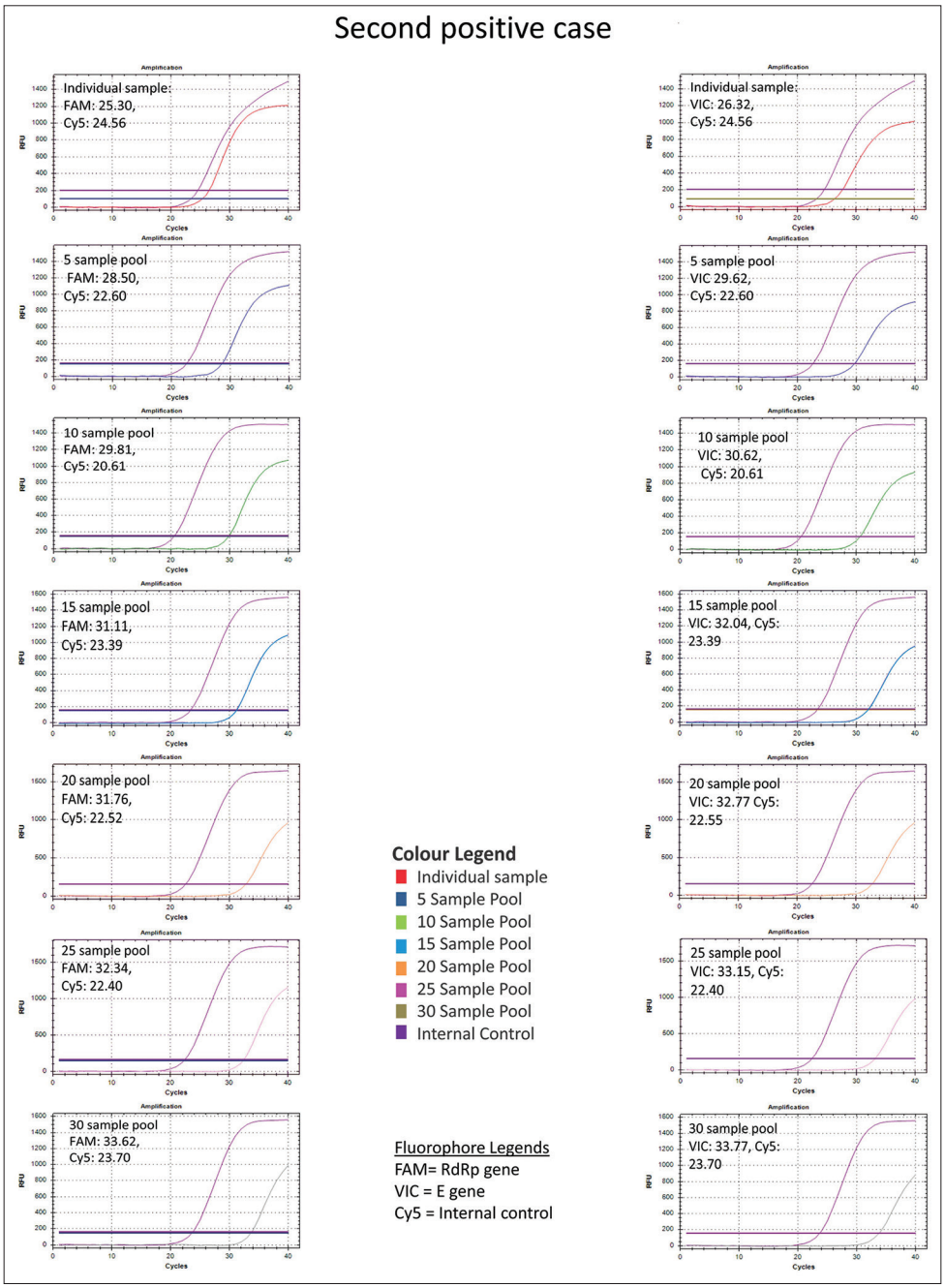

Figure 4: Amplification curves of various pools in the second positive case

and 24.38, respectively, whereas negative control run yielded 22.38 as Ct value for IC [Figure 3 – upper panel]. It was observed that, with two-stage pooling and a pool size of twenty samples (sensitivity of the kit at 95%, positivity rate of 4%), there is 40% reduction in the expected number of tests compared to individual testing.[5-8]

Discussion

Group testing, ever since the idea was put forward by Robert Dorfman in 1943, has undergone manifold modification and has been used in various fields to detect infectious diseases including the last 'swine flu' pandemic (2009 H1N1 Pandemic by H1N1pdm09 virus).[7] This study observed that up to 20-sample pools can effectively be used in mass screening for COVID-19. However, estimating an 'optimal pool size' has to be dealt with caution as it depends on the infection level of the population in that particular geographical area and other factors such as sensitivity of the assay used. Researches show that the lower the prevalence/positivity rate of the disease in that area, greater can be the number of samples pooled together and *vice versa*.[5,8] Currently, in India, positivity rate varies from place to place. While Maharashtra records a high positivity rate of 17.5%, in some states such as Uttarakhand, it is still low (4.1%).[1] Based on this, the optimal group size can be adjusted in different geographical locations. However,

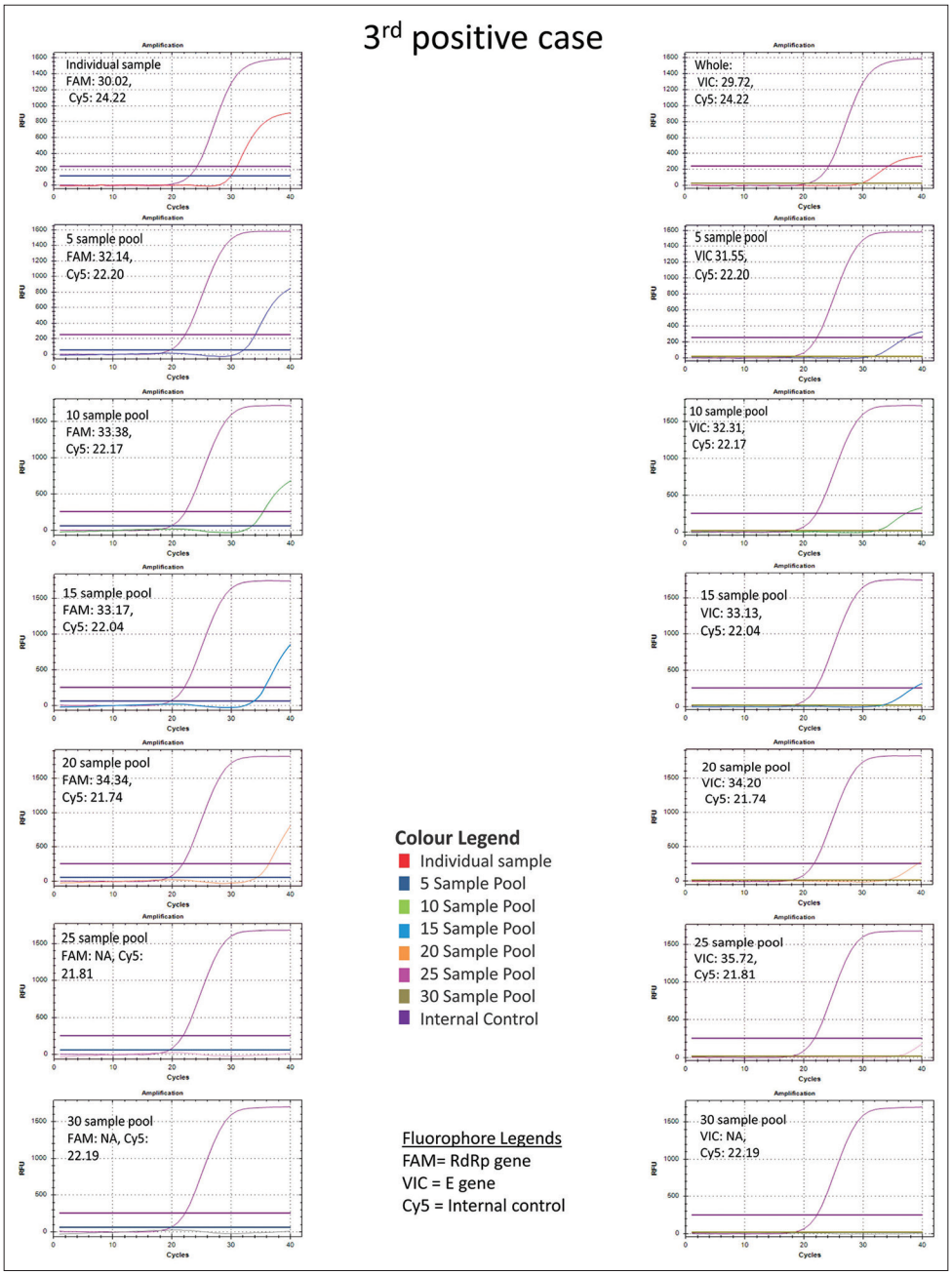

Figure 5: Amplification curves of various pools in the third positive case

some studies prove that for infection levels of 30% and higher, there is no benefit from pooling.[9,10]

A similar study by Lohse *et al.* found a single positive sample from over a range of pool sizes, from 4 to 30 samples, could easily and accurately be identified, which conforms with our findings.[11] Yelin *et al.* also found that a single clinical sample with SARS-CoV-2 RNA can be consistently detected in a pool of up to 32 samples with a linear increase in Ct.[12]

In the first sample, we have observed a decrease in the E-gene Ct value in the pool size of 5 from the testing of single individual sample by −0.77 [Table 1 and Figure 2]. Similar observations were made by Lohse *et al.* which they hypothesised 'were because of the carrier effect of the higher RNA content in pools'.[11]

The most important benefit of group testing is that it has a significant reduction in the number of reactions, thereby saving time, resources and workforce.[5,8,11,13] This study found a 40% reduction in the expected number of tests compared to individual testing.[6] With multi-stage pooling, there can be a further reduction in tests.[8] However, in group testing, there is a risk of borderline positive samples being missed in large pools due to the dilution effect as was noticed in the 3rd positive sample in our study. Therefore, before employing pooling, the sensitivity of the testing technique needs to be characterised.[5,10] Nevertheless, larger studies with more samples and a greater range of pool sizes are recommended to explore the prevalence of false negativity in positive samples with low viral load in group testing.

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Conflicts of interest

There are no conflicts of interest.

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