Viral Transport Media (VTM) Pooling To Scale-Up COVID-19 Diagnostics: Resource Optimization at Population Level Screening

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Research

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Abstract

Background:

The threat of coronavirus disease 2019 (COVID-19) is still looming large on humankind. Pooled-testing can serve as a method of choice for current and future mass surveillance. The aim is to economically benefit in the face of resource-paucity in overburdened public health systems.

Methods:

This study assesses the viability of pooled-testing with viral transport media (VTM) for COVID-19 diagnosis. For this, we designed high-dilution pools using samples with wide-ranging viral loads. The testing employs common TaqMan-based RT-PCR assay considering biological as well as technical replicates.

Results:

We successfully detected a single positive sample in a pool (up to 1/32 dilutions) without loss of sensitivity. The results from biological and technical replicates were found to be satisfactory and reproducible for the objective. We have also discussed multiple variables that can influence the final outcome of pooled testing strategy.

Conclusions:

We show that pooling approach can be efficiently applied for the detection of SARS-CoV-2. We report improved pooling success compared to published studies, even with very low input volume of media, in very high pool-sizes using low-viral load samples. We consider these features to be unique to this study. The study outcomes are encouraging and can be implemented in public health settings to conserve precious resources.

1. Background

The SARS-CoV-2 virus has caused unprecedented damage in recent human history and brought humankind to a complete halt for an entire year 2020 (Zhu et al., 2020). The urgent need of increased diagnostic testing was felt as the COVID-19 epidemic advanced into a pandemic (Sarkar et al., 2020). This was also necessary to distinguish SARS-CoV-2 from closely related other respiratory infections (WHO, 2020).

Different regions across the world are currently experiencing the pandemic in multiple waves and each coming wave has spread more infections and caused more fatalities than the last one. Additionally, more

transmissible variants of the virus, have just begun to emerge and can cause another round of catastrophe (Korber et al., 2020; Volz et al., 2021). The seriousness of the situation can be sensed by the large-scale toll it caused on human lives during the second wave of pandemic in India (Cherian et al, 2021) as well as the concerns of the scientific community over vaccine evasiveness of newer mutants (Garcia-Beltran et al., 2021; Harvey et al., 2021).

The resource-poor lower-middle-income countries, have been suggested to be worst hit due to not only virus, but also their limited testing, isolation and in-patient treatment and care facilities, notwithstanding prevalent socioeconomic inequalities (International, O. 2020; Stewart et al., 2020). Given these challenges, public health agencies across the world, including Indian Council of Medical Research (ICMR) in India, investigated pooled testing as an approach for COVID-19 diagnostics to save on time and resources (Praharaj et al., 2020). Pooled sample testing offers one method to considerably contribute towards enhancing country's testing speed with the available resources. It has been proved to be useful at the peak of pandemic as demonstrated in a few reports (Barak et al., 2020; Garg et al., 2020; Mutesa et al., 2020). Several countries have verified the worth of pooled testing for SARS-CoV-2 and have applied pooled testing to increase testing capability (Europe, 2020; Hogan et al., 2020).

This study presents an application of this method using pooled SARS-CoV-2 suspected nasopharyngeal and/or oropharyngeal (NP/OP) swab specimens in VTM followed by real time-PCR (RT-PCR) testing. The objective of this study was to explore the practicality of detection of SARS CoV-2 positive sample in different pool-sizes in samples with wide-ranging Ct values using commercially available RT-PCR kit .The study also reflects the difference in Ct values of un-pooled and pooled samples

2. Materials And Methods

2.1. Sample acquisition, specimen type and screening for SARS CoV-2

Nasopharyngeal and/or oropharyngeal (NP/OP) swabs suspected with SARS CoV-2, used in this study were submitted to multiple testing centers in Delhi. These specimens were tested by RT-PCR at their respective sites.

The viral transport media (VTM) having NP/OP swab specimens were transported to ICMR-NIP (National Institute of Pathology) on ice. All VTM samples were immediately processed for RNA isolation while leftover VTMs aliquots were refrigerated at -80 ºC for storage.

2.2. Screening of samples for SARS CoV-2: The samples were re-tested to further ascertain their COVID-19 positive/negative status before proceeding to create pooling panels to check for sample deterioration. This screening was done using two different kits, one developed by the ICMR-NIV (National Institute of Virology) two-tube assay based on TaqMan probe-based chemistry, targeting E (envelope gene), RdRp (RNA-dependent RNA-polymerase gene) and ORF1b gene. The other kit is commercially available kit (targeting ORF1ab gene) as per manufacturer's protocol.

In the NIV two-tube assay, the reverse transcription and amplification reaction were performed using Invitrogen SuperScript™ III Platinum® One-Step Quantitative Kit and SARS CoV-2 specific primer–probe mix). Each 25 µL PCR reaction contained 12.5 µL of 2X Reaction mix, 3 µL of PP mix, 0.5 µL of SSIII/Taq Enzyme Mix, 4 µL of RNAse free water and 5 µL of RNA template. The assay was in duplex format and done in parallel: One combined reaction for E (FAM) and RNaseP (VIC) and another reaction for RdRp (FAM) and ORF (VIC). The thermal cycling conditions used were as follows: First, cDNA synthesis at 55 ºC for 15 min. followed by Taq inhibitor inactivation at 95 ºC for 3 min. and then 40 cycles of PCR amplification at 95 ºC for 15 seconds and at 58 ºC for 30 seconds (data collection step) with final extension at 40 ºC for 30 seconds. A sample was considered positive for COVID-19 if the Ct value (cycle threshold) of FAM/VIC channel was less than or equal to 35 with an S-shape amplification curve given the Ct of the positive control RNase P (VIC) should not be higher than 30. The protocol of the respective kits was followed as per manufacturer's instructions.

2.3. Inclusion and exclusion criteria for VTM samples: The VTM samples were selected for pooling following certain inclusion and exclusion criteria (see below).

- VTM samples received as positive and tested positive following in-house screening, were taken as positives.
- VTM samples received as negative and tested negative following in-house screening, were taken as negatives.
- VTM samples tested positive for E gene assay and positive for either of the two NIV kit confirmatory genes (RdRp and ORF1b) or commercial kit (ORF1ab) were construed positive and included for the pooling.
- VTM samples with incongruity in SARS CoV-2 status, on-site and in-house screening were excluded.

2.4. VTM pooling strategy

The VTM pooling strategy was devised considering several points *e.g.* the screening stage Ct values of individual samples, inclusion of biological replicates and technical replicates (**see Additional Table 1**). A total of 184 VTM samples were selected for this study. Out of these, 124 were negative for SARS CoV-2 and 60 samples were positive for SARS CoV-2. Out of these 60, 17 samples showed high Ct (31 to 35) and 5 samples showed very high Ct (above 35).Out of the remaining 38 samples,14 samples showed low Ct (below and up to 25) and 24 samples showed moderate Ct (26 to 30). The Ct values of only confirmatory genes were considered i.e. RdRp and ORF1b or ORF1ab to include a sample into one of these four categories and included.

The Ct value grouping into low, moderate, high and very high concurs with the generally accepted classification in relation to RT-PCR based COVID-19 diagnostic testing. Based on these Ct values, all the positive VTM samples were divided into two groups. Group 1 comprised of samples with low and moderate Ct. Group 2 comprised of samples with high and very high Ct samples.

From group 1, two biological replicate sets were formed (set A and set B). From group 2, two biological replicate sets were formed (set C and set D). It must be noted that in each of the four biological sets, mutually exclusive samples were used (**Table 1**).The VTMs with negative SARS CoV-2 status used, were different between each set in order to introduce sufficient biological variability. The pooling dilutions were as follows: 1/32, 1/28, 1/24, 1/20, 1/16, 2/16, 4/16, 8/16. The specimens used in the study were given random IDs and any personal information related to the samples was not disclosed or used during the experimental design and analysis. The samples were assigned to each set/pool-size after performing randomization 100 times in R Software (R Core Team, 2013).

2.5. Extraction of RNA from VTM pools. RNA was extracted from VTM specimens using QIAamp Viral RNA Mini Kit (Qiagen, Germany, Cat No. 52906). The pools were prepared adding equal volumes from VTMs and according to the pooling plan discussed as per **Table 1**. The final volume in each tube was between 560 to 564µL, from which 560 µL was picked up for downstream RNA extraction processing using column-based manual separation protocol. Purified RNA was eluted in 60 µL of elution buffer and kept in -80 ºC for long-term storage.

2.6. RT-PCR testing of pooled VTMs: Detection of SARS CoV-2 was performed using one-step TaqMan based RT-PCR kit. The kit has ORF1ab as target viral gene and Human β–actin as internal control with load of detection (LOD) of 150 viral copies/mL (for throat swab samples). This kit has sensitivity and specificity of 100% as per the manufacturer and requires 10 µL input RNA. The RT-PCR for each pool was done taking two technical replicates.

RNA was reverse transcribed and amplified using the 2019-nCoV enzyme mix containing Taq DNA Polymerase, reverse transcriptase and UDG. Each 30 µL PCR reaction contained 18.5 µL of 2019-nCoV reaction mix, 1.5 µL of 2019-nCoV enzyme mix and 10 µL of RNA template. The thermal cycling conditions used were cDNA synthesis at 50 ºC for 20 min. followed by initial denaturation at 95 ºC for 10 min. and then denaturation at 95 ºC for 15 seconds and annealing + extension at 60 ºC for 30 seconds for total of 40 cycles. A pool or sample was considered positive for COVID-19 if the Ct value of FAM channel was less than or equal to 38 with an S-shape amplification curve given that the Ct of the positive control (both FAM and VIC channel) should not be higher than 32. The Ct at VIC channel for the internal control (Human β–actin) should also not be higher than 32 with an S-shape amplification curve. The RT-PCR reactions were set up on BioRad CFX96 Touch Real-Time PCR detection system.

3. Results

3.1. RT-PCR of VTM pools: Successful detection of positive amplification was observed, in VTM pools of both, high-V. high (A and B) **Fig. 1**, and low-moderate (C and D) **Fig. 2**Ct groups. In **Fig. 1**, the difference in Ct values observed between set A and set B could possibly be because of biological variation due to different samples being used. Different dilutions within each set (e.g. A32 and A28) contain different samples.

Furthermore, both technical replicates in two biological replicates gave concordant results. For a single case of biological set B (B32), Ct value of 38.8 (borderline positive) against the cut-off of 38 was

observed. The other technical replicate of this pool size did not give amplification curve. This particular pool-size (1/32) was considered to be positive, as the amplification curve was fine despite of borderline Ct value and also the corresponding dilution of both technical replicates of set A gave consistently positive Ct values. Positive amplification in all pools wherever the pool was spiked with positive samples was detected, irrespective of the dilution.

3.2. Comparison of Ct values of samples in pooled testing and individual testing stage: The Ct values of samples were compared at pooled testing and individual testing stage to check if there is any difference observed due to pooling. The categorization of samples into different Ct groups was based on Ct values obtained following in-house screening of VTM samples (where the RT-PCR was done with 140 µL of input VTM).

To make comparison between equal amounts, fresh extraction of RNA was done using the same volume of VTM specimen (560 µL) as the pooled testing stage to avoid input volume bias. For this, 12 individual VTM samples were randomly selected from 1/32 to 1/16 dilutions from each of the four pooling sets (A, B, C and D). The plot in Fig. 3 shows sample-wise comparison of Ct values of ORF1ab gene at pooled testing and individual testing stage. Here, an increase in Ct value change up to6 Ct unit was observed (Fig. 3). The mean increase in Ct was observed to be 1.75 ± 1.01, 95% CI [0.74, 2.76] (calculated using population standard deviation and Z Statistic). This increase in Ct is expected as the pooling dilutes the viral RNA. In two instances, the decrease in Ct values by 0.8 and 1.0 unit from Low-Moderate Ct group (1/16 pool) was observed.

The comparison of Ct values at pooled testing and individual testing stage (from 560 µL of the VTM) are shown in Fig. 4. The panel A is for target gene ORF1ab and expectedly shows positive correlation since the different pooling dilutions have different amount of viral RNA. The panel B is for internal control human β–actin. The dilution does not affect the amount of internal control gene as it is present irrespective of the SARS-CoV-2 status of the samples used in pooling. Together, these observations upheld not just the successful implementation but also emphasize the viability of the pooling experiments presented in this study.

The final reduction in the number of tests is a dynamic outcome of variables considered is shown in Fig. 5. For example, lower test positivity rate suggest larger pool sizes. This approach, though simple, lets the testing of a huge number of individuals with a restricted number of final tests to be done **(Also see additional Figure S3).**

When the data on test positivity rate in India was analyzed (since March 2020 until January 2021), it was observed that the implementation of 32-pool size or even lower pool size based pooled testing could have saved major resources (Fig. 6). The plot underlines the importance of applying pooled testing for saving crucial resources.

Thus, the pooled testing approach can work taking different variables into account, it is recommended to validate it for different settings considering diverse infection prevalence, infrastructure constraints,

sensitivity and specificity of RT-PCR kits among other factors.

4. Discussion

Along with the raging pandemic, the world also battled with the dearth of consumables required for testing because of a sharp rise in the worldwide demand. It added to the inadequate hospital care facilities and diagnostic services, delaying the testing further. Pooled-sample testing is a favorable approach under this situation to test a large population or groups quickly with inadequate resources using a highly sensitive RT-PCR technique. Pooled testing is not a new approach, the basic idea was used to test for syphilis (Dorfman et al., 1943). Among others, it has been used in field for various infections including influenza (Van et al.,2012),Chlamydia (Currie et al., 2004), malaria (Taylor et al., 2010) and HIV (Sullivan et al., 2011). Pooled testing could prove to be a favourable approach to take for both, asymptomatic as well as pre-symptomatic cases along with contact tracing and quarantine.

Whenever the question of resource optimization comes especially in the current scenario, one needs to do away with all the steps that increase the cost of the operation. Researchers have attempted pooling using extracted RNA (Gupta et al., 2020; Khodare et al., 2020; Lim et al., 2020), which in our view is not an optimized pooling strategy and does not reduce the resource costs as compared to VTM pooling. This study attempted pooling of VTM samples before RNA extraction, so that repetitive and not to mention expensive exercise of RNA extraction at the initial stages can be avoided all together. This strategy also saves a lot of RT-PCR reactions. This favours the cost-benefit equation of the entire exercise and leads to a substantial reduction in the consumption of consumables, time and manpower- precious resources in a pandemic situation.

One added benefit of the VTM pooling is that it is amenable to different variations of pooled testing strategy e.g. to collect the NP/OP swabs of whole family in the same VTM tube (swab-pooling) and individualized swabs in separate VTM tube at the same time (for sub-grouping stages). VTM pooling and its different variations can also be easily implemented in the door-to-door and Public Health Clinics (PHCs) settings.

This study, presented the data of VTM specimen pooling for detection of SARS-CoV-2 using RT-PCR. Standardization of pooling experiments was performed using samples with a range of Ct values (from low to very high). Our use of samples with different viral load was deliberate to test the pooled testing in different pool sizes. Pooled testing in eight different dilutions, from 1/32 to 1/2 pool sizes was performed. The biological replicate groups used here, checked for between-sample variation. Further, two technical replicates were used to assess the efficiency and reproducibility of the data.Comparison of Ct values between pooled and individual testing was made to determine whether or not the sensitivity of pooled testing is equivalent to that of individual testing.

Some studies (Gupta et al., 2020) have reported better success with pools of lesser number of samples like 2, 4, and 8. Other reports have drawn attention towards the low sensitivity of pooled testing due to the effect of dilution (Theagarajan, 2020). Our results support the observation that larger pool sizes can

precisely detect a positive sample without any diminished sensitivity. Based on our results, we believe that a positive sample in even more diluted pools can be detected comfortably (above the 1/32 pools e.g. 1/64 dilution) without any compromise on result sensitivity. As of now few countries (Deckert et al., 2020; Yelin et al., 2020) have demonstrated the regular use of pooling at or beyond a batch of 32 samples to be successful in a low infection positivity rate.

Out of 28 pools (for set A and B) and 32 pools (for set C and D), only one replicate showed absence of amplification (in case of low viral load B32 pool), while its technical replicate gave a borderline Ct of 38.8 (with cut-off being 38 according the manufacturer's instructions). Although, for the corresponding dilution in biological replicate group (set A), positive amplification was conclusively detected. Any case (except for one discussed above) where borderline Ct would make the diagnostics of pooled samples difficult was not observed. We suggest that this limitation can be overcome by running the RT-PCR for a few additional cycles (this would help especially in low viral load samples). It is also suggested that RT-PCR testing be performed in replicates to increase sensitivity to avoid false negative results and/or performed with 1/28 pooling dilutions as an upper limit.

For samples where Ct expectedly increased after pooling, the observed mean Ct value for ORF1ab at individual testing stage was 25.3, while at pooled testing stage it was 27.1, that is, an increase in Ct by 1.75.Despite the increase in the Ct, all the values at pooled testing stage did not give rise to false-negative results and were below the cut-off Ct value (i.e., < 38). On the other hand the mean Ct value for human β–actin internal control for both pooled testing and individual testing stage was observed to be 23.9. It makes sense as the amount of internal control remains constant despite different pooling dilutions.

We observed (Fig. 4) lower Ct values in two retested individual samples. The Ct values were found to be decreased by 0.8 and 1 unit. Other reports have also observed this phenomenon (Lohse et al., 2020). As long as differences are small and occur rarely they could possibly be explained by the random experimental variations during pooling, RNA extraction or PCR stage due to small volumes involved in the PCR. Moreover, both of these cases did not cause the samples being labeled as false-negatives as the Ct values were well below the cut-off range.

The Ct values of internal controls from combined data (from individual testing from 140 µL VTM and pooled testing from 560 µL VTM for all twenty samples from four sets) were also compared. Here also, the absence of any correlation was observed as logically expected (**see Additional Figure S2**).

One of the strengths of this study is successful implementation of pooling in light of three factors *viz.* the low amount of input VTM used, the higher pool size tested (up to 1/32) and using samples with low viral. In this study, the lowest amount of VTM used was just 17.5µL in 1/32 pool-size (ranges from 17.5 µL to 35 µL in different pool-sizes) (**Table 1**). To the best of our knowledge, no other study has reported success in pooled testing with input volume of VTM as low as 17.5 µL in high pool size of 1/32 using low viral load samples (Ct range: 30 to 39). It should be noted that using minimal amount of input VTM allows further saving of reagent costs.

Many studies have attempted pooling but have not reported success when all three variables are considered together (low input VTM, low viral load, high pool-size). This not only makes the present study unique and provides significant improvement over other studies but also emphasizes the scope of widespread use of pooled testing. Many studies have reported false negative results when pooled testing using low viral load samples with relatively higher input VTM volumes e.g. with 30 µL in moderate pool-size of 10 (Ct of ORF1ab: 30.7 to 38.5) (Sahajpal et al., 2020), with 60 µL in pools of 5 and 10, even in triplicates (Lim et al., 2020), with 60 µL VTM in low pool-size of 1/5 (Garg A, Ghoshal U et al., 2020), with 25 to 50 µL of input VTM in pool sizes of 5 and 10 (Garg et al., 2020), with an equivalent amount of 40 µL input VTM in pools of 5 and 10 (Praharaj et al., 2020).

Other studies reported pooling success but with comparatively larger input VTM volume (500/8 = 62.5 µL of input VTM) and low pool-size (1/8) in low-to-moderate Ct samples (~ 17–22, ~ 25–27 and 29-31.8) (Yelin et al., 2020), with 50 µL of input VTM in pools of 5 (Abdalhamid et al., 2020), with 50 to 80 µL of input VTM in low pool sizes of 8 and 5 (Barak et al., 2020).

We also did encounter a failure to detect amplification in one technical replicate at higher dilution of 1/32. Overall we have been able to detect positive sample in higher dilutions in low viral load sample pools in corresponding biological replicate group, especially with such low amount of input VTM. Our results improve upon the set guidelines and highlight the scope of pooled testing for better mass surveillance.

Hierarchical pooled testingis a common pooled testing and sub-grouping method. Using an open-access tool, describing pooled testing and retesting procedures for hierarchical group testing (Black et al., 2015), we looked at the feasibility of the pooled testing approach with respect to different variables. Several variables were considered to calculate their potential in reduction of testing resources. The variables include pool size, sensitivity/specificity of the RT-PCR kit used, stages of pooling, sub-pooling at the 2nd stage pooling and most importantly the test positivity rate (**Additional Table 2 and Additional Figure S1**).

In view of current pandemic the entire world has been reeling under, the significance of faster diagnostics is of paramount importance. There is an urgent need to screen large groups such as schools, universities, prisons and large workplaces in order to prevent them becoming infection hotspots. Different districts/community pockets and urban-rural settings may show different prevalence. Pooled testing in such eligible settings may lead to early detection of infected people thus help controlling community spread. Since disease prevalence is low now (< 5% in India at the time of communicating this paper), it's the appropriate time to implement this strategy so that large population/community groups are screened *en masse* and positive cases isolated immediately to prevent transmission.

Moreover, the real possibility of a new wave caused by mutant strains, including the delta variant (B.1.617.2) and delta-plus variant (B.1.617.2.1/ (AY.1)) is currently making headline along with B.1.617.3 and B.1.1.318 which are already present in India (Cherian et al., 2021). These strains are reported to be more infectious and/or more fatal (Garcia-Beltran et al., 2021; Harvey et al., 2021). These rising mutant

strains have started spreading to many countries plausibly due to resuming of international movement – reiterating the importance of pooled testing.

5. Conclusion

It is evident from the present study that direct VTM pooling approach can be efficiently applied for the detection of SARS-CoV-2 employing RT-PCR. It was demonstrated that the pooled testing shows no loss of sensitivity and works comparable to individual testing. The study showed success in pooled testing using low volume of input VTM in high pool sizes using low-viral load samples. Conclusions drawn from our results are of paramount importance to design and implement policy and practices of the mass-surveillance in the face of current as well as future pandemics. This is vital, particularly in times of increased demand of testing for an infectious disease in a large population. We strongly feel that even after when the pandemic is considered to be controlled, pooled testing has a big role to play to prevent future isolated and sporadic bursts of infections across the country and the world.

List Of Nonstandard Abbreviations

VTM: Viral Transport Medium

SARS-Cov-2: Severe Acute Respiratory Syndrome Coronavirus-2

COVID-19: Coronavirus Disease 2019

WHO: World Health Organization

ICMR: Indian Council of Medical Research

RT-PCR: Real Time-Polymerase Chain Reaction

Declarations

Disclaimer: This study was undertaken on clinical samples for the sole objective of demonstration of feasibility and efficiency of VTM pooled testing with intention neither to validate any RT-PCR test kit nor to provide any positive or negative recommendation on its use in the testing scheme.

Ethics approval and consent to participate: NIP-ICMR, which is the place of research for this work was one of the designated centers for SARS CoV-2 RT-PCR kit validation by ICMR, the national organization responsible for deciding guidelines during pandemic. All biological specimen were thus received for the purpose of testing under the above-mentioned mandate. The analysis is based on completely de-identified specimen and no personal information related to the subjects were accessed or used in this work. Written informed consent by the source public testing agency/lab was taken from each subjects.

Consent for publication: Not applicable

Availability of data and material: Data and materials are available upon request to the corresponding author

Competing interests: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authorship contribution: The contribution of all authors are as follows:

PG: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Visualization, Writing- Original draft preparation. **FA:** Writing- Original draft preparation, Validation. **JS[1]:** Writing- Original draft preparation, Investigation. **JS[2]:** Writing- Original draft preparation. **SS:** Experimental help. **AA:** Resources management. **KKR:** Writing - Review & Editing. **NG:** Writing - Review & Editing. **NZE:** Supervision, Resources, Writing - Review & Editing. **UA:** Conceptualization, Project administration, Writing - Review & Editing. All authors have read and approved the final version of the manuscript. (**JS[1]:** Jyoti Sharma**, JS[2]:** Jasmine Samal)

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Tables

Table 1. Pooling strategy applied to different Ct groups and biological replicates.

Pooling set	Ct Group	Samples in VTM pool		Amount of VTM taken from each samples (in µL)	Total amount of VTM (in µL)	Total no. of samples in pool
		Positive samples	Negative samples			
A32	High-V High	1	31	17.5	560	32
A28	High-V High	1	27	20	560	28
A24	High-V High	1	23	23.5	564	24
A20	High-V High	1	19	28	560	20
A16	High-V High	1	15	35	560	16
A8	High-V High	2	14	35	560	16
A4	High-V High	4	12	35	560	16
B32	High-V High	1	31	17.5	560	32
B28	High-V High	1	27	20	560	28
B24	High-V High	1	23	23.5	564	24
B20	High-V High	1	19	28	560	20
B16	High-V High	1	15	35	560	16
B8	High-V High	2	14	35	560	16
B4	High-V High	4	12	35	560	16
C32	Low-Moderate	1	31	17.5	560	32
C28	Low-Moderate	1	27	20	560	28
C24	Low-Moderate	1	23	23.5	564	24
C20	Low-	1	19	28	560	20

	Moderate					
C16	Low-Moderate	1	15	35	560	16
C8	Low-Moderate	2	14	35	560	16
C4	Low-Moderate	4	12	35	560	16
C2	Low-Moderate	8	8	35	560	16
D32	Low-Moderate	1	31	17.5	560	32
D28	Low-Moderate	1	27	20	560	28
D24	Low-Moderate	1	23	23.5	564	24
D20	Low-Moderate	1	19	28	560	20
D16	Low-Moderate	1	15	35	560	16
D8	Low-Moderate	2	14	35	560	16
D4	Low-Moderate	4	12	35	560	16
D2	Low-Moderate	8	8	35	560	16

Figures

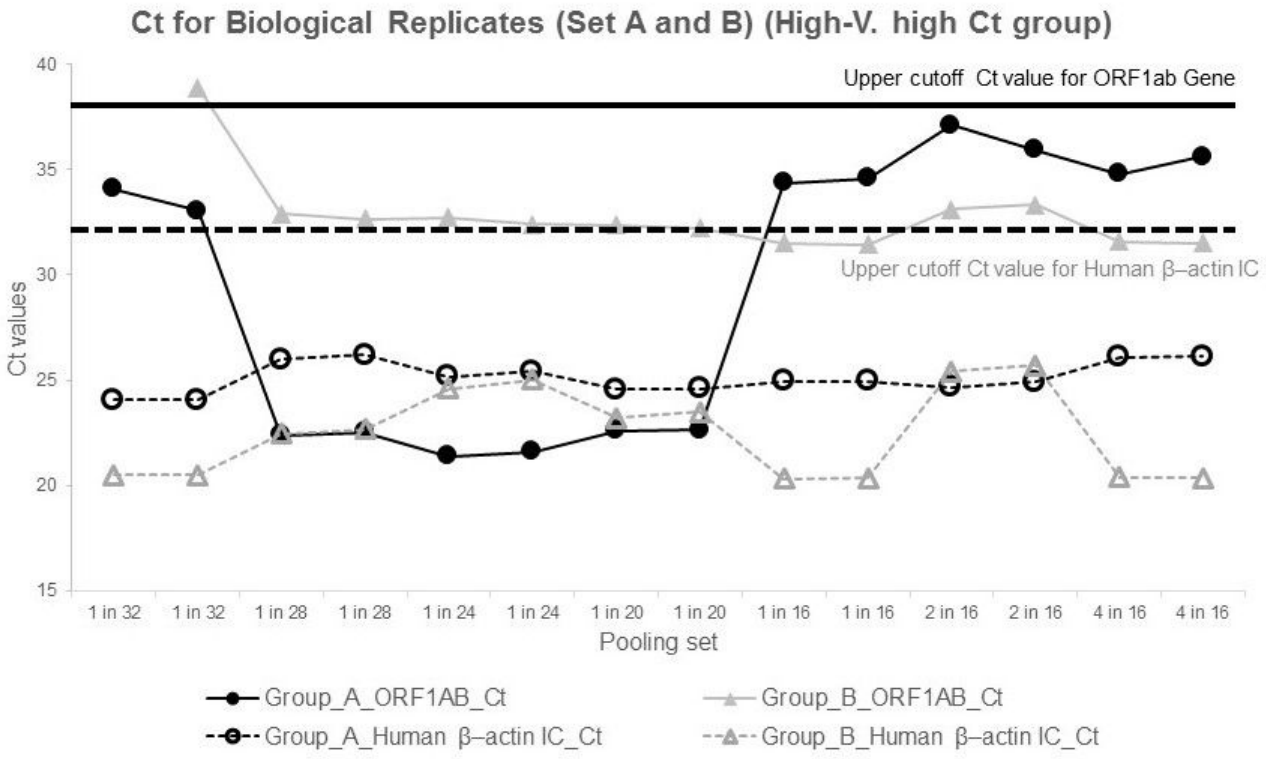

Figure 1

Pooled RT-PCR testing results for biological replicates, set A and set B (High-V. high Ct group). The plot shows the Ct values after pooled testing in different pooling dilutions for s belonging to high-very high Ct group. The horizontal solid and dotted line refers to the cut-off for the ORF1ab (Ct ≤38) and human β–actin internal control (Ct ≤32) genes, respectively.

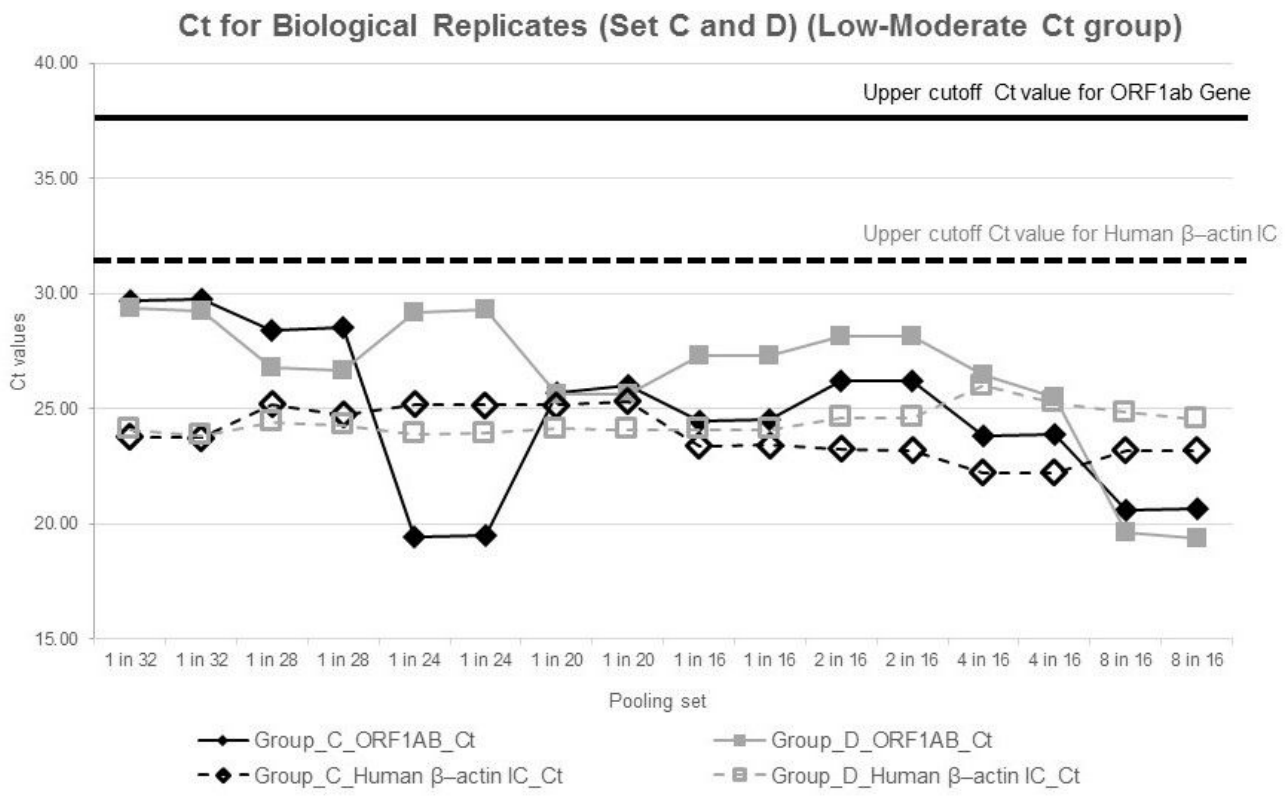

Figure 2

Pooled RT-PCR testing results for biological replicates, set C and set D (Low-Moderate Ct group). The plot shows the Ct values after pooled testing in different pooling dilutions for samples belonging to Low-Moderate Ct group. The horizontal solid and dotted line refers to the Ct cut-off for the ORF1ab (Ct ≤38) and human β–actin internal control (Ct ≤32) genes, respectively.

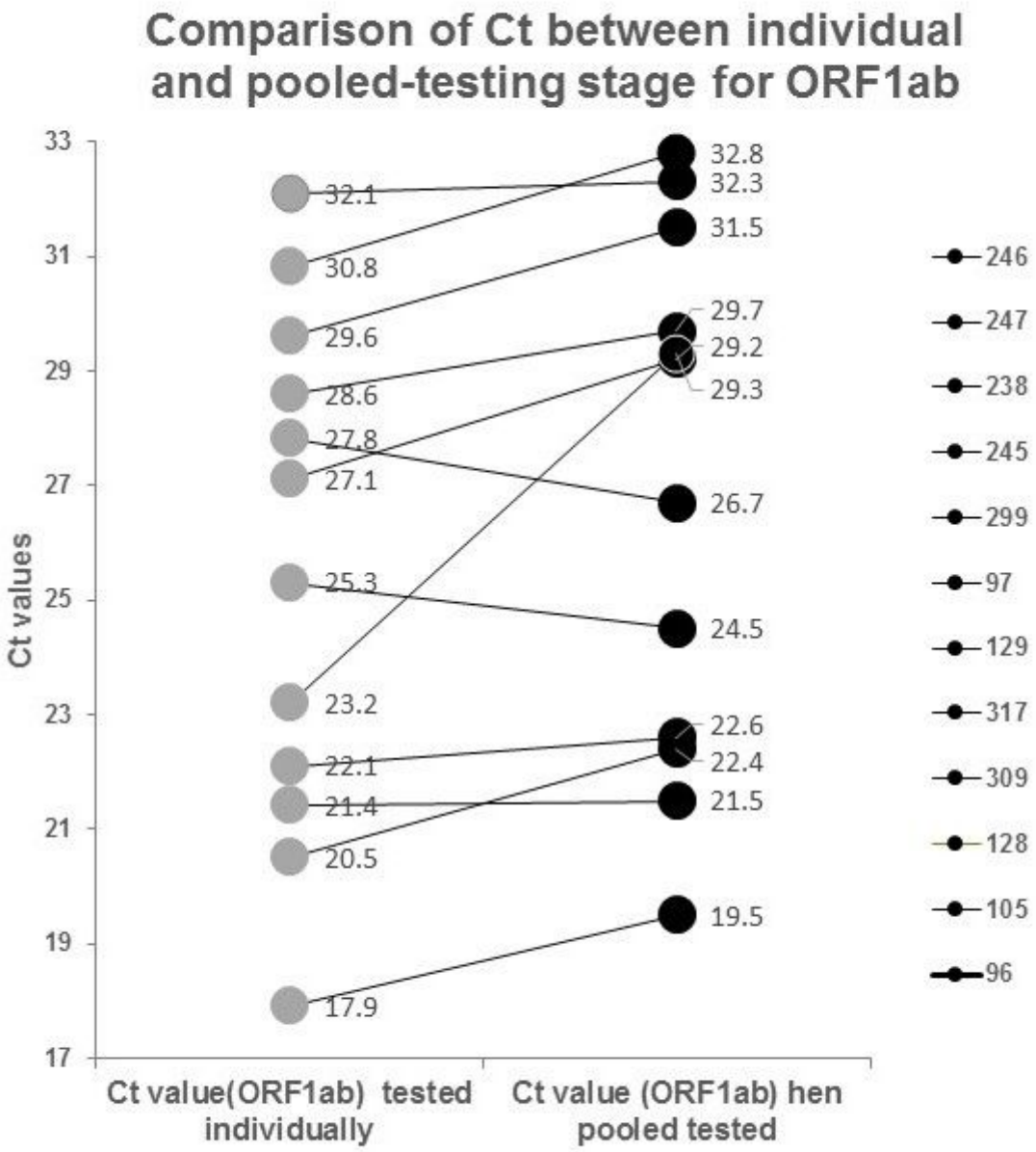

Figure 3

Ct comparison in pooled testing and individual testing stage for viral target and internal control gene. The graph compares the Ct values of samples for target gene ORF1ab at pooled testing and individual testing stage. Samples show an expected rise in Ct when tested in pools. The mean increase in Ct was found to be 1.75.

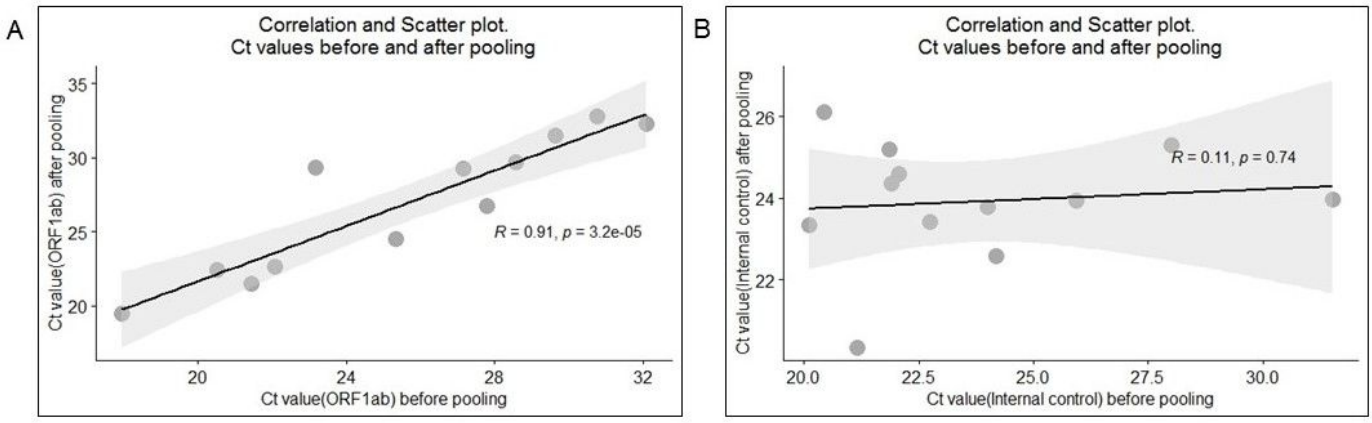

Figure 4

The scatter plot and correlation between Ct values before and after pooling for (A) ORF1ab gene (BGI kit) and (B) Internal control human β–actin. In plot A the Ct values before and after pooling shows a strong positive correlation (R = 0.91, p-value = 3.2 x 10-6). The plot B shows the absence of any correlation (R = 0.11, p-value = 0.74) between Ct values of human β–actin which is an internal control in RT-PCR reaction.

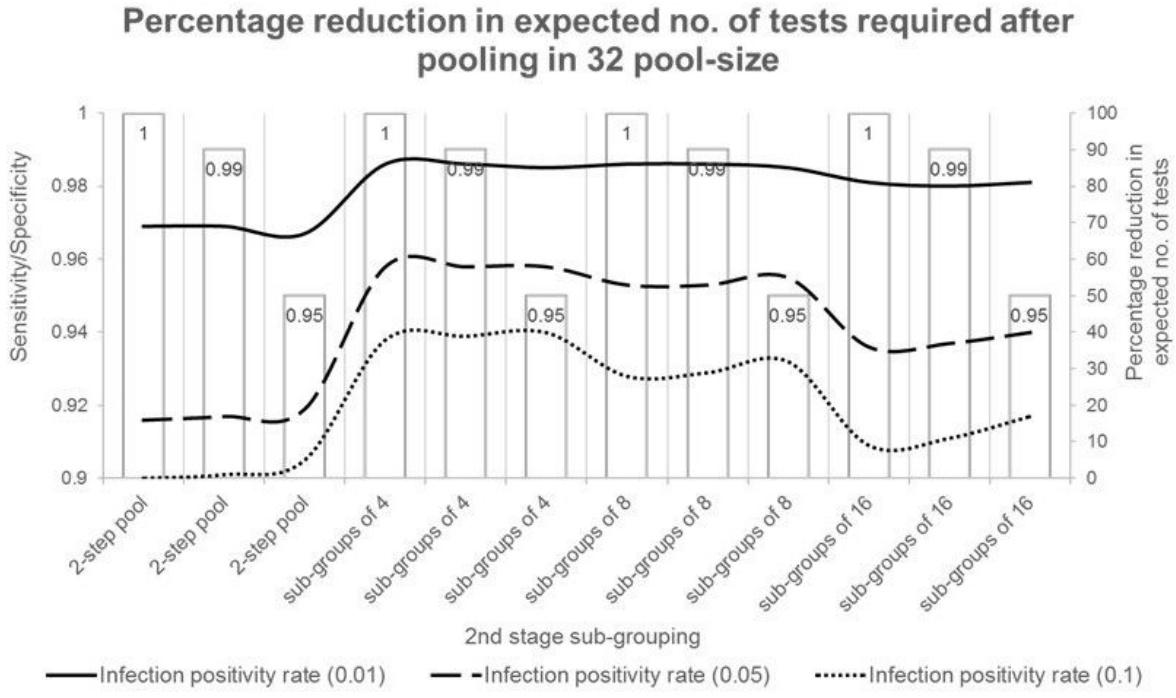

Figure 5

Effect of different variables on the final reduction in the consumption of the diagnostic tests required. The plot shows the effect of variables like sensitivity/specificity of the kit used, 2nd stage sub-grouping and infection positivity rate on the final reduction of expected number of tests in case of pool size of 32. The calculations were done using a publically available tool (Black et al., 2015). Effect of sensitivity/specificity of the kit used, 2nd stage sub-grouping and infection positivity rate on final reduction of expected number of tests in case of pool size of 16. Three different bar-plots refer to the different sensitivity/specificity of kit to be used for pooling (100%, 99% and 95%) on left y-axis. Three points refer to the %reduction in expected no. of tests on right y-axis. The x-axis refers to the pool design. First three bar-plots are for 2-step pooling and rest are for 4x4 and 8x2 sub-pools for 3 stage pooling plan.

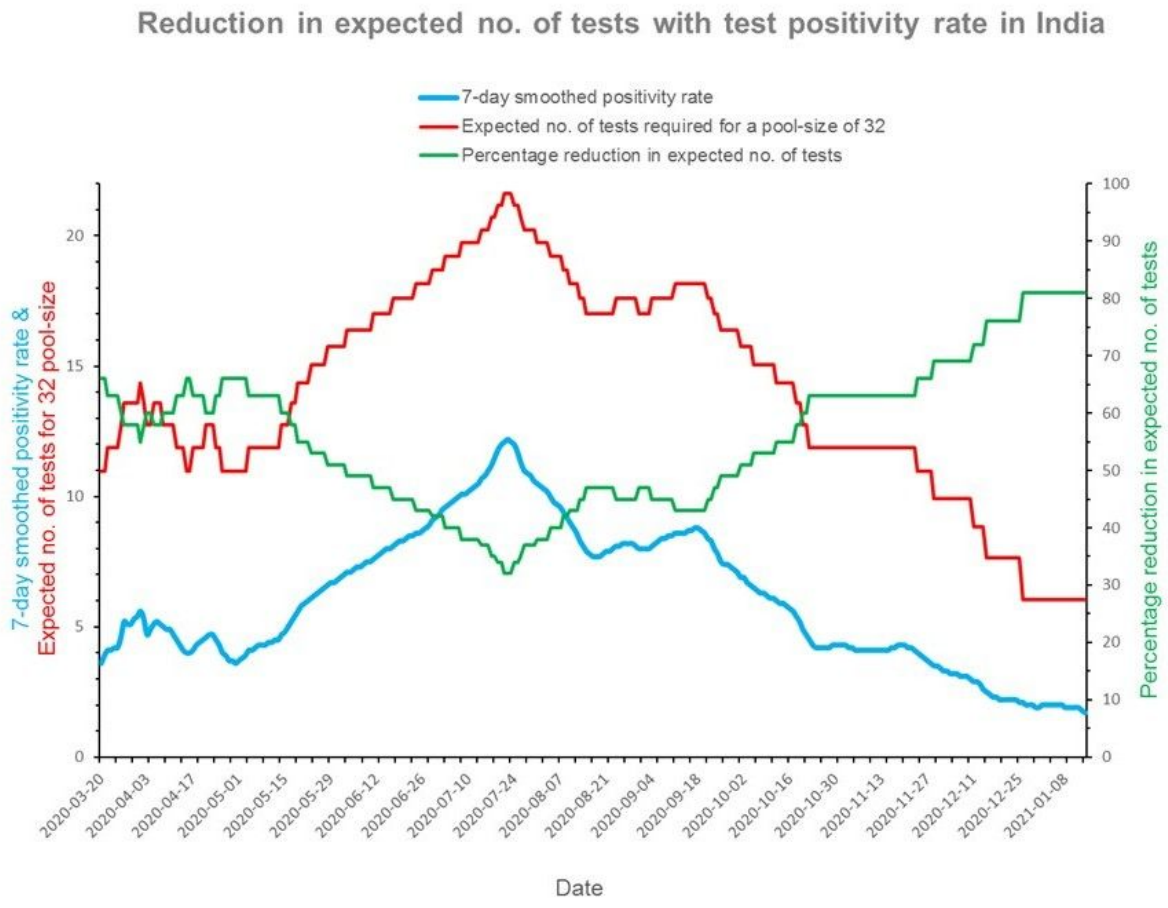

Figure 6

Application of pooled testing as affected by the infection prevalence in India. The graph shows India's data on test positivity rate (in blue) from 20th March till 15th Jan 2020. The positivity rate ranges from 1.7 to 12.7 %. The y-axis shows the test positivity rate, smoothed over 7 day period. The graph takes the following parameters as constant: pool size = 32, sensitivity/specificity of the RT-PCR kit used = 0.99, 3-stage pooling and 2nd stage pooling with eight batches of 4 sample pools. The calculation is based on non-overlapping window of 0.5 % in positivity rate. Red and green lines refer to the lower bound of the

positivity rate window. The calculations are done using a publically available tool (Black et al., 2015). (Data source: https://ourworldindata.org/coronavirus/country/india?country=~IND).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigureS1.jpg
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- SupplementaryFigureS3.jpg
- SupplementaryTable1.xlsx
- SupplementaryTable2.xlsx
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