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A pooled RT-PCR testing strategy for more efficient COVID-19 pandemic management

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ABSTRACT

Objectives: Reverse transcription polymerase chain reaction (RT-PCR) testing is indispensable in management of the coronavirus disease 2019 (COVID-19) pandemic. However, with the emergence of new variants of severe acute respiratory syndrome coronavirus-2, the cause of COVID-19, the screening capacity of RT-PCR testing is overburdened, and new strategies and capabilities need to be established. One option is pooled RT-PCR testing.

Design: This study used various mixtures of COVID-19 samples known to be negative and positive, and investigated the impact of pool size and mixture level on final cycle threshold (Ct) values. More specifically, 5, 10 and 20 negative samples were combined with one, two or three low Ct or high Ct positive samples.

Results: Average baseline Ct and numbers of high and low Ct samples in the pool were found to be the main drivers of the final Ct value, making detectability easier. Pool size was not significantly associated with final Ct, but was suggestive.

Conclusions: A pooled RT-PCR testing strategy does not reduce the sensitivity of RT-PCR, and thus provides a practical way to expand RT-PCR screening capacity in pandemic management. The pool size was not found to be significant, so it is recommended that a pool size of 20 would be a practical number to reduce the time taken to obtain the results and the cost of RT-PCR testing.

Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the cause of coronavirus disease 2019 (COVID-19), emerged in Wuhan, China in December 2019, and has since spread around the world. By mid-May 2020, more than 90 million people had been tested, more than 4.5 million people had been infected, and there had been more than 300,000 deaths globally. While the USA has experienced the greatest numbers of cases and deaths, new epicentres of the pandemic are emerging, such as Russia, Brazil and India, and it is highly likely that further epicentres will emerge.

By the end of August 2021, approximately 2.5 billion tests had been conducted and more than 216 million positive cases had been reported. When considering those countries that report both the number of tests conducted and the number of positive cases identified, the COVID-19 positivity rate is seen to oscillate between 4% and 10%, with a current mean of 6.4% (Figure S1, see online supplementary material).

In fighting this pandemic, chest computed tomography is one of the first-line testing modalities for patients who present to the healthcare system with symptoms, especially respiratory symptoms (Li and Xia, 2020). In addition, the World Health Organization has provided guidelines for COVID-19 genetic-based testing using nucleic acid amplification tests, such as reverse transcription polymerase chain reaction (RT-PCR) (WHO, 2020). RT-PCR tests are conducted in designated laboratories by trained personnel, and test accuracy is affected by sample quality (Lippi et al., 2020) – regardless of whether the sampling is oropharyngeal or nasopharyngeal (Carver and Jones, 2020) – and RNA degradation (Tang et al., 2020). Antibody test kits are used mainly as supplementary tools to the RT-PCR approach to diagnose past infections using body fluids (e.g. blood); however, these tests have less favorable diagnostic measures (Tang et al., 2020), their timing is highly critical, and repeat tests are necessary (Beeching et al., 2020). The COVID-19 diagnostics report published by the National University of Singapore, Saw Swee Hock School of Public Health, describes many commercial

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and non-commercial COVID-19 diagnostic tests (He et al., 2020). However, very few of these tests were presented with corresponding sensitivity and specificity measures, and unrealistic characteristics such as 100% sensitivity and 100% specificity were reported (Yap et al., 2020). Chan et al. (2020) reported that RT-PCR has 95% sensitivity.

Pooling the samples for COVID-19 testing has been explored by several researchers. Ben-Ami et al. (2020) found that sensitivity was maintained when using a pool size of eight samples. Praharaj et al. (2020) investigated pooled sampling with pool sizes of five and 10 samples, and found no significant loss of sensitivity, even for samples with low viral loads. Garg et al. (2021) used 40 negative samples and 10 positive samples in their pooled sample testing, and compared seven commercial kits: the TRUPCR Kit, TaqPath Kit, Allplex Assay and BGI RT-PCR showed perfect agreement, whereas the Fosun, LabGun and Patho Detect Kits achieved 90%, 85% and 75% accuracy for low viral load samples, respectively. Kocak (2020) showed the probabilistic properties of pooling COVID-19 samples for RT-PCR testing in terms of resulting false-positive and false-negative rates.

This study presents a more comprehensive pooled-sampling strategy for COVID-19 RT-PCR testing, with carefully selected and independent COVID-19 positive samples with varying viral loads, varying pool sizes and negative–positive combinations.

Materials and methods

This pooled-sampling strategy employed the following steps:

(1) Sets of five, 10 and 20 COVID-19 samples that were tested as negative using RT-PCR were identified. These sets formed the negative base.
(2) Positive samples with low cycle threshold (Ct) and high Ct values were identified.
(3) One, two or three positive samples were added to the negative pools, and the resulting Ct values were reported.
(4) Each combination was repeated five times.

Table S1 (see online supplementary material) illustrates the details of the combinations tested, showing a total of 21 combinations.

In brief, 2.5 µL was taken from nasopharyngeal and oropharyngeal swab samples for RT-PCR in Bio-speedy vNAT transfer tubes (Bioeksen, Istanbul, Turkey). According to the manufacturer's protocol, 5 µL of 2X Prime Script Mix and 2.5 µL of 2X Prime Script Mix were reacted, with a total volume of 10 µL. RT-PCR tests were performed using a Biorad CFX96 (Bio-Rad Laboratories, Inc., California, USA) in accordance with the conditions in Table S2 (see online supplementary material). The recommended threshold level for CFX96 Touch instruments (Bio-Rad Laboratories, Inc., California, USA), 200 RFU, was used in the analysis. After examining the shape of the amplification curves, if a sample was given a Cq value by the instruments' software and the curve was sigmoidal, the result was decided using the Cq value. Non-sigmoidal curves were considered negative. If a sample was given a Cq value but the curve was not sigmoidal, the result was recorded as negative. While sigmoidal curves with Cq-HEX (IC) $\leq$30 were included in the analysis, samples with non-sigmoidal curves or Cq-HEX>30 were repeated.

Analysis of covariance models were constructed, where Ct measures for the positive samples ('baseline Ct') were added to the model as the covariate, and the numbers of negative samples, low Ct positive samples and high Ct positive samples were added as factors of interest. When there was only one positive sample in the pool, its Ct value was used as the baseline Ct. When there were two or three positive samples, the average value was used as the baseline Ct value. For high Ct positive samples in the pool, the median Ct was 18.33 (range 14.17–19.71). Similarly, for low Ct positive samples in the pool, the median Ct was 29.00 (range 27.30–31.49).

The pooled-sample Ct ('final Ct') was the primary outcome variable in these models. These models assessed the significance of baseline Ct and varying combinations of samples for prediction of the final Ct measurement in the pooled sample.

Results

This multivariable model found a linear association between baseline Ct and final Ct, with a one-unit-higher baseline Ct resulting in a one-unit-higher final Ct, on average. The number of high Ct samples in the pool was significantly associated with final Ct, suggesting that every additional high Ct sample added to the pool decreased final Ct by one unit, controlling for baseline Ct (Figure 1); similarly, every additional low Ct sample added to the pool decreased final Ct by approximately 2.6 cycles, on average, controlling for baseline Ct (Figure 2).

Although the interaction between baseline Ct and number of high Ct samples in the pool was not very strong (P=0.014), the interaction between baseline Ct and number of low Ct samples in the pool was highly significant (P=0.0007), as shown in Figure 2.

Pool size was not highly significant but was suggestive of final Ct, controlling for baseline Ct (0.065) and number of high and low Ct samples in the pool. In addition, there was no significant interaction with baseline Ct (P=0.65), as shown by almost parallel prediction lines for different pool sizes in Figure 3.

Discussion

This study showed that RT-PCR tests are highly sensitive regardless of how many positive samples are included in a pool, and the level of dilution by the number of negative samples forming the pool. The number of negative samples was kept constant when changing the pool size in order to keep the background (i.e. dilution level) stable, and one, two or three positive samples with differing strength mixtures (e.g. all high Ct, all low Ct, or a combination) were added. Despite the changes, RT-PCR on the pooled sample was able to detect COVID-19 positivity in all combinations, except in one repeat of the scenario with a pool size of 20 with one low Ct sample (Ct=30), and two repeats of the scenario with a pool size of 10 with one low Ct sample (Ct=29.7) and with two low Ct samples (Ct=29.7 and 27.7), and finally in two repeats of the scenario with a pool size of five with one low Ct sample (Ct=27 and 30). None of the pooled samples including at least one high Ct sample failed to detect the virus. Negative pools were tested with five repeats, and all repeats tested negative, as expected.

Overall, of the 44 samples with baseline Ct <20, only four (8.3%) resulted in final Ct <20. Thirty-six (75%) samples had final Ct between 20 and 25, and the remaining eight (16.7%) samples had final Ct between 25 and 30. Similarly, of the 36 samples with baseline Ct between 20 and 25, 24 (67.7%) had final Ct between 20 and 25, and the remaining 12 (33.3%) samples had final Ct between 25 and 30 (Table S3, see online supplementary material).

With pooling, it is expected that Ct values will increase due to mixing with negative values. However, interestingly, for some combinations, especially in pools mixing negative samples with low Ct samples, final Ct values decreased, suggesting improved detectability (Figure 4).

Table 1 shows some specific cases with better detectability with pooling.

This study also investigated the impact of variability of baseline Ct on final Ct. When considering high Ct samples, no significant effect was observed; however, when at least two low Ct samples were included in the pool, there was a significant association between Ct variability and final Ct (Figure S1, see online supplemen-

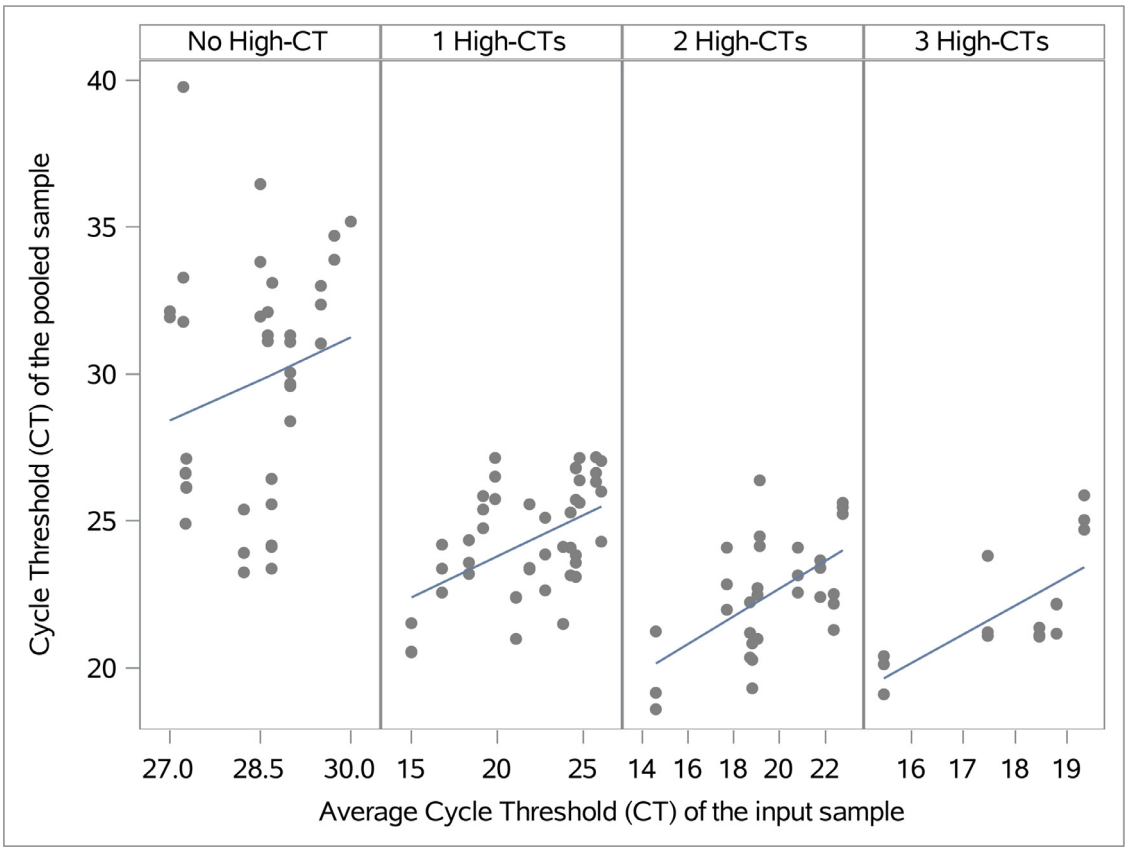

Figure 1. Association of high cycle threshold (Ct) numbers in the pool with final Ct.

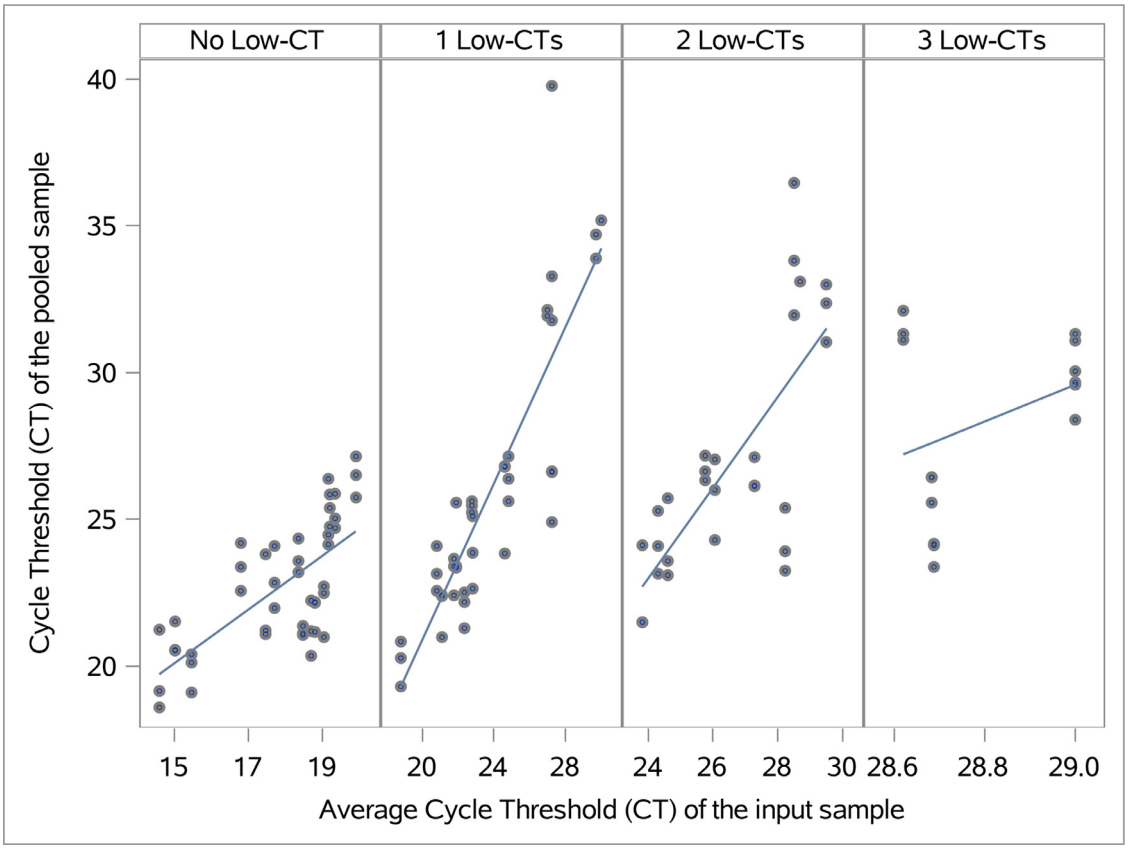

Figure 2. Association of low cycle threshold (Ct) numbers in the pool with final Ct.

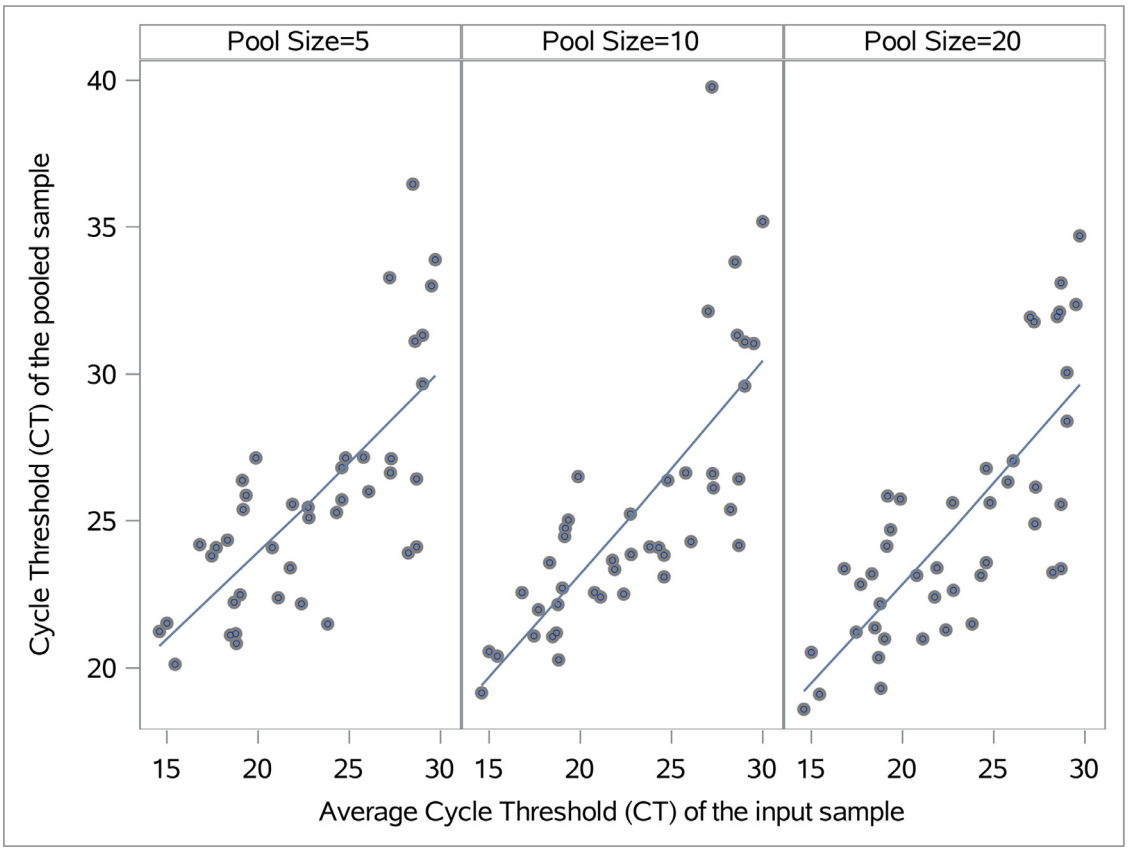

Figure 3. Association of baseline cycle threshold (Ct) numbers with pool size.

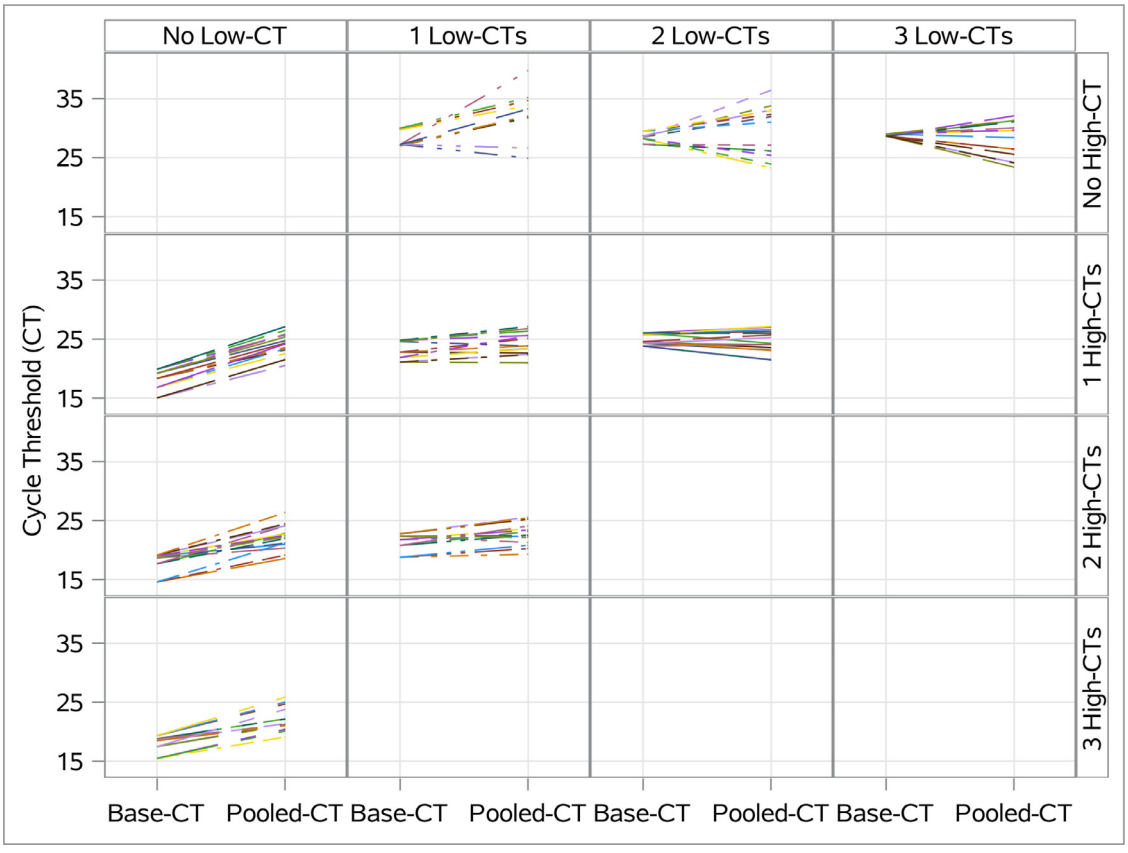

Figure 4. Changes in cycle threshold (Ct) values from baseline to pooled evaluations.

Table 1
Selected cases with pooled cycle threshold (Ct) lower than average baseline Ct.

No. of negative samples	No. of high Ct samples	No. of low CT samples	Baseline Ct-1	Baseline Ct-2	Baseline Ct-3	Average baseline Ct	Final Ct	Difference
20	0	3	27.22	29.23	29.61	28.687	23.38	-5.307
20	0	2	27.22	29.23	.	28.225	23.26	-4.965
5	0	3	27.22	29.23	29.61	28.687	24.11	-4.577
10	0	3	27.22	29.23	29.61	28.687	24.16	-4.527
5	0	2	27.22	29.23	.	28.225	23.9	-4.325
20	0	3	27.26	27.3	31.49	28.683	25.56	-3.123
10	0	2	27.22	29.23	.	28.225	25.38	-2.845
20	0	1	27.26	.	.	27.26	24.9	-2.36
20	1	2	15.02	27.22	29.23	23.823	21.49	-2.333
5	1	2	15.02	27.22	29.23	23.823	21.5	-2.323
5	0	3	27.26	27.3	31.49	28.683	26.44	-2.243
10	0	3	27.26	27.3	31.49	28.683	26.44	-2.243

tary material), where increased variability (i.e. more diverse low Ct samples included) resulted in better detectability (i.e. lower final Ct).

To assess the reproducibility of Ct measurements, a separate experiment was conducted using positive samples alone. Pools of 2, 5, 10 and 20 positive samples, grouped as high Ct samples and low Ct samples (a total of eight independent scenarios), were used. From each pool, Ct was measured with five repeats. One of the practical messages for this supplementary analysis was that the standard deviation of the Ct values averaged approximately 0.32 (range 0.115–0.56). This means that the Ct measurements were fairly stable with repeat tests in a pooled strategy, and were not expected to change beyond approximately 0.6 cycles (based on error margin of 2 standard deviations). Another interesting finding from the supplementary analysis was that for higher standard deviations of baseline Ct in the high Ct scenarios, the standard deviation of final Ct decreased, while the opposite was true for the low Ct pools (Figure S2, see online supplementary material)

One weakness of the experimental design was that individual and pooled samples were not processed on the same plate simultaneously. This was partly because each pool needed to be described in terms of negative samples, and high or low Ct positive samples. The authors are currently working on simultaneous processing of individual and pooled samples on the same plate at their testing centre.

Another possible weakness of this study was that the authors were unable to test larger pool sizes (e.g 50 and 100) due to the logistical difficulties of establishing such pools given the need to keep the amount of sampling material taken from each sample uniform.

The way in which nasopharyngeal/oropharyngeal samples are taken is known to be among the most important factors affecting RT-PCR results. As the samples sent to the authors' centre for testing are taken by different health personnel from many institutions, this can be considered as a negative factor. Also, since the outbreak of the pandemic, many changes have been observed in the SARS-CoV-2 genome, and it is not known how this will affect the present results. In addition, Ct values may be affected in frozen and thawed samples.

As governments are forced to lift COVID-19 restrictions for the workforce, transportation and schools, the need for pooled and pod-testing is increasing. Although such tests are typically performed using rapid test kits, there is no doubt that the need for pooled-sample RT-PCR testing will also increase with current and potential future variants of this coronavirus. A pooling approach will reduce the burden on testing laboratories and result in cost-saving. As mentioned earlier, in every pool of 20 samples, one positive sample is expected based on the positivity rate profile globally, which may differ by region based on RT-PCR testing strategies. It is clear that in any mass-testing strategy, the positivity rate will decrease as the net cast will catch more asymptomatic cases, the majority of whom will not be infected. Therefore, as the pandemic restrictions are lifted, with plans for and more and more testing, a pooled-sample strategy offers a solution to reduce the burden on testing laboratories and keep the cost of increased testing programmes at affordable levels to central governments as well as local administrations. In these analyses, the pool size was a suggestive but non-significant predictor of final Ct, and was not significantly associated with baseline Ct; in fact, the weak association between pool size and final Ct was negative, suggesting that increasing pool size resulted in a slightly lower final Ct. If one assumes a linear trend, increasing the pool size from 10 to 20 would decrease final Ct by 0.6, slightly more than half a cycle on average, controlling for the other key factors in the model. In addition, as mentioned earlier, failure to detect positivity in a pooled sample, although rare, was experienced in all three pool sizes used in this study (five, 10, and 20 samples). Therefore, the authors recommend that a pool size of 20 should be used for pooled-sampling RT-PCR for mass testing.

Conclusions

RT-PCR testing has high sensitivity for pooled sampling using pools containing various combinations of negative and positive COVID-19 samples. With the lifting, or relaxation, of pandemic restrictions, the need for testing will increase, and this additional burden can be managed through carefully structured pooled sampling with RT-PCR.

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Conflict of interest statement

None declared.

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Ethical approval

As this study used blinded COVID-19 tests with no protected health information, ethical approval was not required.

Author contributions

MK generated the research idea, established the experimental design, conducted data analyses and prepared the manuscript. AA and AGOD conducted the experiment, helped with the literature search and prepared the manuscript. GO helped with manuscript preparation.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2021.12.328.

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