narra j

Short Communication

Comparison of RT-PCR cycle threshold values between individual and pooled SARS-CoV-2 infected nasopharyngeal swab specimens

Handa Yani[1*], Toh D. Yuan[2], Aridamuriany D. Lubis[1], Lia K. Iswara[3] and Inke ND. Lubis[1]

[1]Department of Pediatric, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia; [2]Department of Biomedical Engineering, College of Design and Engineering, National University of Singapore, Singapore; [3]Department of Microbiology, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia

*Corresponding author: handa566725@gmail.com

Abstract

The molecular reverse transcription-polymerase chain reaction (RT-PCR) testing of respiratory tract swabs has become mandatory to confirm the diagnosis of coronavirus disease 2019 (COVID-19). However, RT-PCR tests are expensive, require standardized equipment, and relatively long testing times, and the sample pooling method has been introduced to solve this issue. The aim of this study was to compare the cycle threshold (Ct) values of the individual sample and pooled sample methods to assess how accurate the pooling method was. Repeat RT-PCR examinations were initially performed to confirm the Ct values for each sample before running the pooled test procedure. Sample extraction and amplification were performed in both assays to detect ORF1ab, N, and E genes with a cut-off point value of Ct <38. Overall, there was no difference in Ct values between individual sample and pooled sample groups at all concentrations (p=0.259) and for all pooled sizes. Only pooled size of five could detect the Ct value in the pooled samples for all concentration samples, including low-concentration sample (Ct values 36 to 38). This study highlighted that pooled RT-PCR testing strategy did not reduce the quality of individually measured RT-PCR Ct values. A pool size of five could provide a practical technique to expand the screening capacity of RT-PCR.

Keywords: COVID-19, diagnostic, RT-PCR, pooling method, Ct value

Introduction

Since the coronavirus disease 2019 (COVID-19) pandemic, the reverse transcription-polymerase chain reaction (RT-PCR) test of respiratory tract swabs has become mandatory to confirm the diagnosis of COVID-19 [1]. The RT-PCR test targets the ORF 1a, ORF 1b, S, and N genes of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), capable of detecting <10 copies/genome per reaction [1-6]. This is recommended by the World Health Organization (WHO) and provides sensitivity of 90% to 100% and specificity of 100% [7]. However, RT-PCR tests are not only expensive, but they also require standardized equipment, sample preparation and testing procedures that require trained personnel, and long testing times [2]. This poses a challenge, especially in developing countries with a lack of laboratory facilities and resources [2]. To address these issues, the sample pooling method has been introduced [2,7,8]. The concept of the sample pooling method is collecting multiple swab samples in one tube (a pool) before testing them. This approach is an alternative for testing large numbers of COVID-19 samples as it requires less time, expenses, and reagents [2,9-14]. As the prevalence of COVID-19 decreases but the need for screening increases, the need for pooling is essential [3,7].

https://narraj.org/

Received: March 22, 2024 - Accepted: June 17, 2024 - Published online: June 19, 2024

Studies recommend that in areas where the positivity rate is lower than 2%, sample pooling should be viable [15,16]. Another study also reported the successful large-scale screening pooling of asymptomatic populations [17]. In fact, the trend of pediatric cases during peak COVID-19 cases, which was the first year of the pandemic, was still less than 3% in North Sumatra, Indonesia [18]. Therefore, pooled testing is considered feasible to be applied in North Sumatra. The use of pooled testing with 5 to 25 pooled samples will reduce the number of tests required approximately by 75%, resulting in significant cost savings [2,14,19] Studies have shown that pooling of 5 or 10 nasopharyngeal specimens can detect positive SARS-CoV-2 [15,20-22]. However, there is a possibility that pooling of specimens may cause dilution and lead to false-negative results, affecting the accuracy of the test [15,16,23-25]. Nevertheless, there have been no studies in North Sumatra, Indonesia, analytically comparing the Ct values between individual and pooled samples. Therefore, the aim of this study was to compare the Ct values generated from the individual sample and pooled sample methods to assess how accurate the pooling method was.

Methods

Study setting and procedure

This comparative study was conducted at the Microbiology Laboratory, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia, from May to June 2023. The samples were individuals who underwent nasopharyngeal swab for RT-PCR testing. The inclusion criteria in this study were nasopharyngeal swab samples obtained both in the asymptomatic SARS-CoV-2 infected individuals and COVID-19 patients collected at the Microbiology Laboratory, Faculty of Medicine, Universitas Sumatera Utara with a Ct value <38, while samples with Ct values ≥38 or undetected were excluded from the study. A total of 25 samples were used in this study with varying Ct values (from low to high concentrations) and were randomly allocated in each pooled (**Table 1**). The samples were further classified based on Ct values into 16 to 20, 21 to 25, 26 to 30, 31 to 35, and 36 to 38. The samples were also categorized as high concentration if their Ct value was <25, medium if the Ct value was between 25 and 30 and low if the Ct value was >30. A RT-PCR examination was initially performed to confirm the Ct values for each individual sample before running the pooled test procedure. RNA extractions were performed using the automatic instrument (AllSheng Auto-Pure 32A Nucleic Acid Purification System, Hangzhou, China) and utilized MagBind RNA extraction kit (Maccura Biotechnology, China, Lot-1122023, Ref-GN7101907) before the RT-PCR test was performed.

Materials and laboratory test

Samples utilized in this study were nasopharyngeal swabs collected in the Microbiology Laboratory, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia. The RNA extraction was carried out using an automatic instrument (All Sheng Auto-Pure 32A Nucleic Acid Purification System, Hangzhou, China) and utilized MagBind RNA extraction kit (Maccura Biotechnology, China; Lot-1122023, Ref-GN7101907). The RT-PCR machine (Roche Light Cycler®96, Mannheim, Germany) was employed for amplification procedure. Reagent for amplification procedure consisted of 17 µL master mix NC (ORF lab/N) PCR and 3 µL enzyme (ORF lab/N) of Maccura SARS-CoV-2 fluorescent PCR kit (Maccura Biotechnology, USA; Lot 0822961, Ref.-EGN7103109).

At the extraction stage, 300 µL of pooled sample was mixed into 12 µL of reagent (10 µL mag-bind RNA and 2 µL internal control). At the amplification stage, 5 µL of extracted RNA was tested for RT-PCR.

Pooling strategy

Pooled testing was carried out by grouping samples based on the intended group size. It was a two-stage testing algorithm. In the first stage, samples were divided into separate groups of n samples each, and each group was tested. In this study, we pooled 300 µL of positive samples (marked in red) and 1200 µL of negative samples (300 µL per tube of negative samples, marked in blue) into 10 mL tubes for the pooled test with a group size of five. Similarly, 300 µL of positive samples and 2100 µL of negative samples were pooled for group size of eight, 2700 µL of negative samples for group size of ten, 4200 µL of negative samples for group size 15, 5700 µL of negative

samples for group size 20, and 7200 µL of negative samples for group size 25 (300 µL per tube of negative samples) in 10 mL tubes (**Figure 1**). In the second step, the pooled samples (solution A to solution F) were extracted and RT-PCR amplified. A negative result implied that all samples within the group were negative, whereas a positive result indicates that at least one sample in the group was positive. In the second stage, samples from each group with positive results are individually tested.

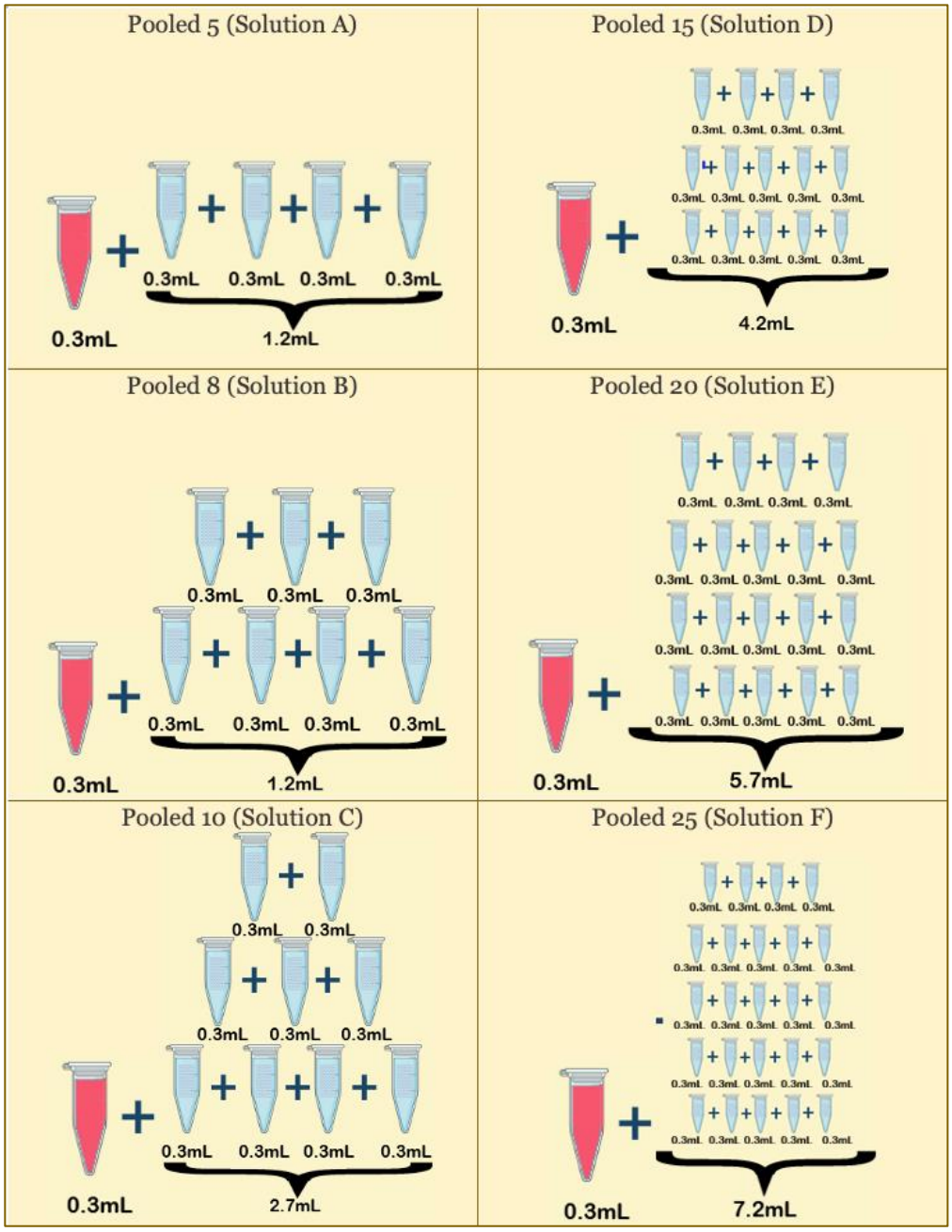

Figure 1. Illustration of the solution in each pool. Each of the pool was extracted and RT-PCR amplified.

Statistical analysis

The data analysis was conducted using the Statistical Package for the Social Sciences (SPSS) version 21 (IBM, New York, USA). The normality of the Ct values was tested using the Shapiro-Wilk test. The paired Student t-test for normally distributed data and the Wilcoxon test for non-normally distributed data were used to compare the Ct values between groups. A *p*-value of <0.05 was considered statistically significant.

Results

Comparison of Ct values between individual and pooled methods across various sample sizes and viral concentrations

The distribution of individual Ct values, pooled Ct values, and the differences/shifts between the two methods are presented in **Table 1**. It demonstrated the shift variations between individual Ct values and pooled Ct values among sample concentrations. The biggest shift was 15.25 reported on a 25-sample pooled in high viral concentration (Ct value 21–25) (**Table 1**). Our data indicated that there was no difference in Ct values between individual sample and pooled sample groups for all pooled sizes (from 5 to 25 pooled sizes) (**Table 2**).

Table 1. Distribution of Ct values in individual and pooled samples

Parameter	Pooled size	Individual Ct value	Pooled Ct value	Ct value difference
Ct 16–20 (high concentration)	5	16.14±3.62	27.11±3.85	10.97
	8	16.92±2.49	32.18±3.24	15.25
	10	17.68±2.83	32.55±3.42	14.87
	15	18.35±3.53	29.53±3.53	11.18
	20	19.89±4.91	30.54±2.77	10.56
	25	16.14±4.73	29.18±3.93	13.04
Ct 21–25 (high concentration)	5	21.03±2.90	32.65±3.62	11.62
	8	21.59±2.87	35.58±2.84	13.98
	10	22.45±3.91	34.05±3.93	11.60
	15	23.97±2.73	35.37±2.94	11.39
	20	24.48±3.78	28.46±3.58	3.98
	25	21.03±4.90	36.28±3.99	15.26
Ct 26–30 (moderate concentration)	5	26.49±3.91	31.59±3.79	5.11
	8	26.49±2.82	32.87±3.36	6.38
	10	27.15±3.78	32.04±3.98	4.89
	15	27.15±2.15	33.19±3.25	6.04
	20	27.99±1.95	29.08±3.89	1.09
	25	27.99±3.12	29.71±2.48	1.72
Ct 31–35 (low concentration)	5	32.97±2.95	31.19±2.94	-1.77
	8	32.97±3.32	34.55±1.59	1.59
	10	33.39±2.71	32.41±2.65	-0.98
	15	33.39±2.31	35.13±4.85	1.74
	20	34.35±2.17	36.63±3.94	2.28
	25	34.35±3.94	36.82±3.63	2.47
Ct 36–38 (low concentration)	5	36.54±2.17	36.96±2.64	0.42
	8	37.27±3.15	Negative (38.49±3.65)	1.23
	10	36.01±3.46	Negative (38.86±3.94)	2.85
	15	36.96±2.45	Negative (39.12±3.83)	2.17
	20	37.71±3.82	Negative (39.43±3.39)	1.72
	25	36.53±2.97	Negative (39.22±3.57)	2.69

Table 2. Differences in Ct values between pooled samples and individual samples based on pooled size

Pooled size	Individual Ct value	Pooled Ct value	*p*-value
Pooled 5	26.63±8.36	31.90±3.52	0.121 [a]
Pooled 8	27.05±8.24	34.73±2.49	0.165 [a]
Pooled 10	27.33±7.56	33.98±2.83	0.056 [b]
Pooled 15	27.96±7.40	34.46±3.49	0.054 [a]
Pooled 20	28.88±7.22	32.82±4.90	0.061 [a]
Pooled 25	27.21±8.64	34.24±4.51	0.074 [a]

[a] Analyzed using paired Student t-test
[b] Analyzed using Wilcoxon test

The overall shifts of Ct values between pooled and individual samples across the viral concentration (initial Ct values) are presented in **Figure 2**. It indicated that the differences in Ct values between individual and pooled samples were higher as the viral concentration in the samples was lower (higher Ct values).

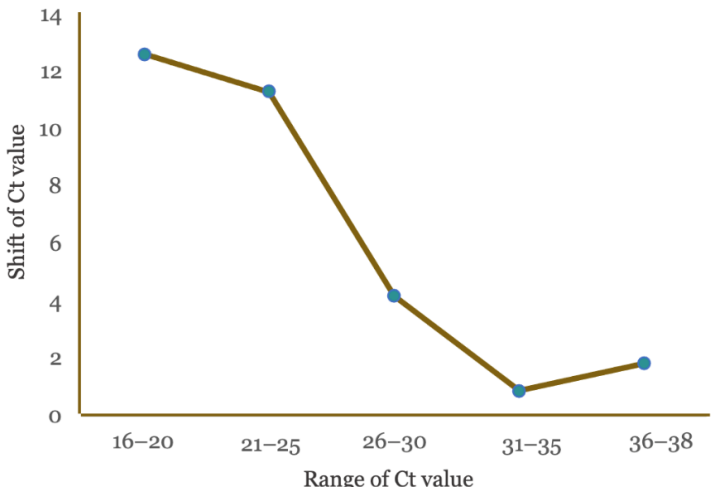

Figure 2. The overall difference of Ct values between individual and pooled groups based on viral concentrations.

Comparison of Ct values between individual and pooled samples

The comparison of Ct values between individual samples and pooled samples, based on the viral concentration (classification of Ct values), is presented in **Table 3**. There were no significant differences between the groups of Ct values 16–20, 21–25, 26–30, and 31–35, with p=0.657, p=0.461, p=0.053, and p=0.053, respectively. The group with Ct values of 36–38 yielded a p=0.043, indicating a significant difference between individual and pooled groups. However, there was no significant difference between groups in the overall test (p=0.259).

Table 3. Comparison of tested Ct values between individual and pooled samples based on viral concentrations

Viral concentration	Group	Mean Ct value	p-value
Ct value 16–20 (high concentration)	Individual	17.79±1.44	0.657[a]
	Pooled	30.38±2.02	
Ct value 21–25 (high concentration)	Individual	22.70±1.49	0.461[a]
	Pooled	33.22±2.88	
Ct value 26–30 (moderate concentration)	Individual	33.56±0.67	0.053[a]
	Pooled	33.41±1.67	
Ct value 31–35 (low concentration)	Individual	33.56±0.63	0.053[a]
	Pooled	33.41±2.26	
Ct value 36–38 (low concentration)	Individual	36.89±0.62	0.043[b]
	Pooled	22.70±0.90	
Overall	Individual	29.08±7.88	0.259[b]
	Pooled	30.09±3.27	

[a] Analyzed using paired Student t-test
[b] Analyzed using Wilcoxon test

Discussion

The sample pooling strategy for SARS-CoV-2 testing allows for increased detection rates while maintaining sensitivity. The result of our study indicates the pooled sample size was not in line with the shift in Ct values between the pooled and individual samples. Theoretically, as the size of pooled samples increases, the difference in Ct values between pooled and individual samples should decrease [22,26]. However, this was not seen in this study, as the delta Ct between groups that was consistent with the size of pooling was in the Ct value classification of 31 to 35 only.

In samples with Ct values less than 30 (high to moderate virus concentration), the 20-sample-pooled method demonstrated the smallest shift in Ct values between pooled and individual samples. These differences are less than those of the 5-sample-pooled at Ct value classifications 16 to 20 (high concentration) and were much lower than those of the 5-sample-pooled at Ct value classifications 21 to 25 (high concentration) and Ct value classifications 26 to 30 (medium concentration). Although, for samples in the Ct value classifications 31 to 35, the

shift in Ct values in the 20-sample-pooled was the second highest with a delta value of 2.281 (**Figure 3**).

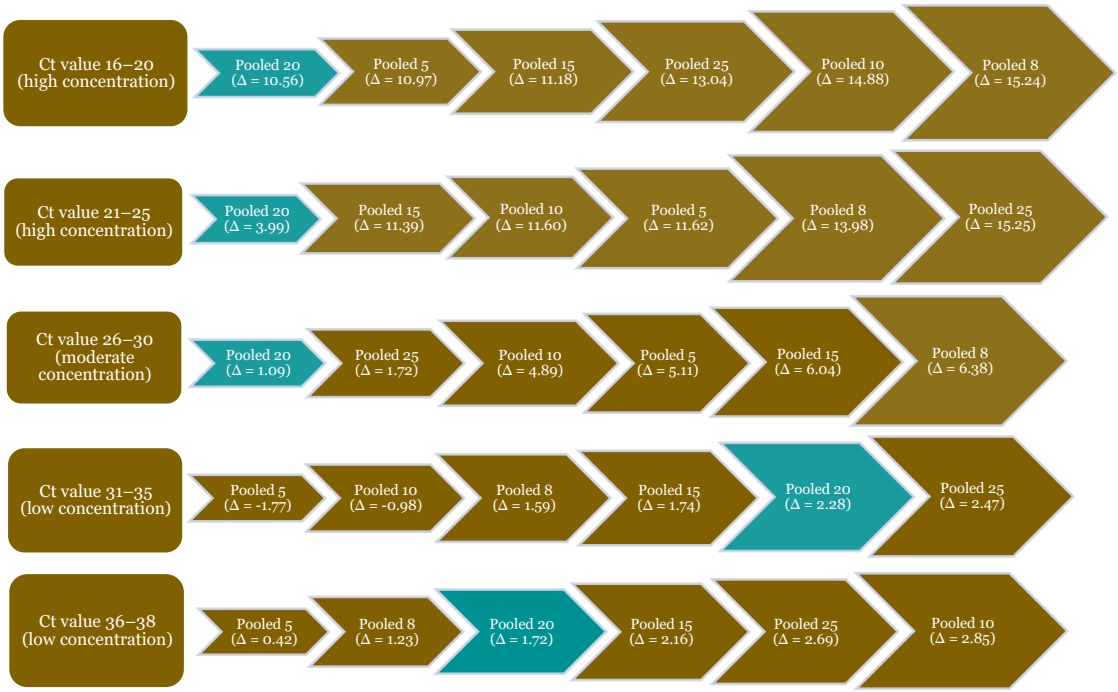

Figure 3. The increasing order of delta/difference Ct values between individual samples and pooled samples at each viral load concentration. The dark teal highlighted area represents the pool with the lowest delta Ct value.

In samples with low concentrations (Ct value more than 36), our study showed that the pooled size of 5 samples consistently yielded the lowest shift in Ct values between pooled and individual samples. This finding is in concordance with a demonstration that the identification of positive samples with low Ct values (<36) was achieved in pool sizes of 5 and 10 samples [27]. However, the false-negative rate was higher when testing samples with high Ct values (>36).

Our study also found that in the Ct value classification of 31 to 35, the Ct value of the pooled group was lower than those in the individual group, specifically in 5-and 10-sample pooled methods. Theoretically, Ct values are expected to increase due to dilution with negative values in pooling. However, similar results were obtained by another study, indicating that the lower the final Ct value, the better the detection [28]. In our study, although the 20-sample pool method had the lowest shift, the 5-sample pool method was the only pool group that could be detected (Ct value <38) for low-concentration samples (Ct value classification of 36 to 38). This is similar to the results of the previous studies, which stated that at 1% infection rate, the optimal pool size is 11 and when the infection rate was 10%, the optimal pool size was reduced to 4 [26,29]. In our study with a positivity rate of 2–3%, the 5-pooled samples proved to be in accordance with the formula of these studies as the best optimal pool size [21,26,29,30]. Thus, the 5-sample pooled method is the best method for pool RT-PCR without loss of sensitivity (**Table 2**).

The difference in Ct values between pooled and individual samples in overall pooling sizes and Ct classification was no greater than 15.25 (i.e., pooled 25 and Ct classification of 21 to 25). Furthermore, the results of this analytical study examining the comparison of the average Ct value between the pooled sample and the individual sample using the Wilcoxon test showed no significant difference between groups in the overall test (p=0.259). This indicates that pooling has been analytically validated in diagnosing COVID-19.

The strength of this study was that we elaborated the comparison of pooling sizes and Ct values classification between individual sample and pooling sample in more detail, whereas previous studies only classified the Ct value of a sample as low or high concentration. These findings might provide reliable support for the use of 5-sample-pooled method in mass RT-PCR

swab testing, which could reduce the burden on testing laboratories and result in cost-saving of approximately 75% [2].

Conclusion

There is no difference in Ct values between the individual sample group and the pooled sample group. However, our data suggested that applying pooled five samples would be the best approach. Nevertheless, further study with bigger sample size would be critical before this strategy could be used as a standard for COVID-19 mass testing.

Ethics approval

The Research Ethics Committee at the Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia approved the study protocol (approval No. 1065/KEP/USU/2021).

Acknowledgments

The authors would like to thank all participants included in this study.

Competing interests

The authors declare that there are no conflicts of interest.

Funding

This study did not receive any specific external funding.

Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

How to cite

Yani H, Yuan TD, Lubis AD, *et al*. Comparison of RT-PCR cycle threshold values between individual and pooled SARS-CoV-2 infected nasopharyngeal swab specimens. Narra J 2024; 4 (2): e765 - http://doi.org/10.52225/narra.v4i2.765.

References

1. Corman VM, Landt O, Kaiser M, *et al*. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill 2020;25(3):2000045.
2. Deckert A, Bärnighausen T, Kyei NN. Simulation of pooled-sample analysis strategies for COVID-19 mass testing. Bull World Health Organ 2020;98(9):590-598.
3. Sethuraman N, Jeremiah SS, Ryo A. Interpreting diagnostic tests for SARS-CoV-2. JAMA 2020;323(22):2249-2251.
4. Hartenian E, Nandakumar D, Lari A, *et al*. The molecular virology of coronaviruses. J Biol Chem 2020;295(37):12910-12934.
5. Wang D, Jiang A, Feng J, *et al*. The SARS-CoV-2 subgenome landscape and its novel regulatory features. Mol Cell 2021;81(10):2135-2147.
6. Banko A, Petrovic G, Miljanovic D, *et al*. Comparison and sensitivity evaluation of three different commercial real-time quantitative PCR kits for SARS-CoV-2 detection. Viruses 2021;13(7):1321.
7. Xu W, Li X, Dozier M, *et al*. What is the evidence for transmission of COVID-19 by children in schools? A living systematic review. J Glob Health 2020;10(2):1-12.
8. World Health Organization. Coronavirus disease (COVID-19): Situation report - 207. WHO 2020. Available from: https://www.who.int/publications/m/item/situation-report---207. Accessed: 10 June 2023.
9. Esteves E, Mendes AK, Barros M, *et al*. Population wide testing pooling strategy for SARS-CoV-2 detection using saliva. PLOS ONE 2022;17(1):1-13.
10. Long QX, Tang XJ, Shi QL, *et al*. Clinical and immunological assessment of asymptomatic SARS-CoV-2 infections. Nat Med 2020;26(8):1200-1204.

11. Mastrianni D, Falivena R, Brooks T, *et al*. Pooled testing for SARS-CoV-2 in hospitalized patients. J Hosp Med 2020;15:538-539.
12. Majid F, Omer SB, Khwaja AI. Optimising SARS-CoV-2 pooled testing for low-resource settings. Lancet Microbe 2020;1(3):e101-e102.
13. Bogere N, Bongomin F, Katende A, *et al*. Performance and cost-effectiveness of a pooled testing strategy for SARS-CoV-2 using real-time polymerase chain reaction in Uganda. Int J Infect Dis 2021;113(1):355-358.
14. Wacharapluesadee S, Kaewpom T, Ampoot W. Evaluating the efficiency of specimen pooling for PCR-based detection of COVID-19. J Med Virol 2020;92(10):2193-2199.
15. Mahmoud SA, Ibrahim E, Thakre B, *et al*. Evaluation of pooling of samples for testing SARS-CoV- 2 for mass screening of COVID-19. BMC Infect Dis 2021;21(1):360.
16. Augenblick N, Kolstad J, Obermeyer Z, Wang A. Pooled testing efficiency increases with test frequency. Proc Natl Acad Sci USA 2022;119(2):1-7.
17. Ben-Ami R, Klochendler A, Seidel M, *et al*. Large-scale implementation of pooled RNA extraction and RT-PCR for SARS-CoV-2 detection. Clin Microbiol Infect 2020;26:1248-1253.
18. Dicky D, Marpaung K, Nainggolan IR, *et al*. The trend of paediatric cases during the first year of the COVID-19 pandemic in North Sumatra, Indonesia. IJID Reg 2023;8(1):S18-S21.
19. Prakash S, Prakash O, Mishra H, *et al*. Feasibility, efficiency and effectiveness of pooled sample testing strategy (pooled NAAT) for molecular testing of COVID-19. Indian J Med Res 2021;153:227-232.
20. Praharaj I, Jain A, Singh M, *et al*. Pooled testing for COVID-19 diagnosis by real-time RT-PCR: A multi-site comparative evaluation of 5- & 10-sample pooling. Indian J Med Res 2020;152(1):88-94.
21. Shukla S, Upadhyay V, Maurya VK. Evaluating the efficiency of specimen (sample) pooling for real-time PCR based diagnosis of COVID-19. Indian J Med Microbiol 2021;39(3):339-342.
22. Mintsa NR, Zoa AS, Mewono L, *et al*. Could pooled samples method affect SARS-CoV-2 diagnosis accuracy using BGI and Sansure-Biotech RT-PCR kits used in Gabon, Central Africa?. Plos One 2022;17(1):1-12.
23. Haque A, Pant AB. Efforts at COVID-19 vaccine development: Challenges and successes. Vaccines (Basel). 2020;8(4):739.
24. Shen K, Yang Y, Wang T, *et al*. Diagnosis, treatment, and prevention of 2019 novel coronavirus infection in children: Experts' consensus statement. World J Pediatr 2020;16(3):223-231.
25. Kar SK, Verma N, Saxena SK. Coronavirus infection among children and adolescents. Coronavirus Disease 2019 (COVID-19) 2020;71-79.
26. Polage CR, Lee MJ, Hubbard C, *et al*. Assessment of an online tool to simulate the effect of pooled testing for SARS-CoV-2 detection in asymptomatic and symptomatic populations. JAMA 2020;3(12):1-4.
27. Handous I, Hannachi N, Marzouk M, *et al*. Pooling nasopharyngeal swab specimens to improve testing capacity for SARS-CoV-2 by real-time RT-PCR. Biol Proced Online 2021;23(1):19.
28. Ayaz A, Demir AGO, Ozturk G, Kocak M. A pooled RT-PCR testing strategy for more efficient COVID-19 pandemic management. Int J Infect Dis 2022;116:1-6.
29. Hanel R, Thurner S. Boosting test-efficiency by pooled testing for SARS-CoV-2—Formula for optimal pool size. PloS one 2020;15(11):1-10.
30. Lim KL, Johari NA, Wong ST, *et al*. A novel strategy for community screening of SARS-CoV-2 (COVID-19): Sample pooling method. PloS one 2020;15(8):1-10.