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Original article

Retrospective analysis of SARS-CoV-2 RNA detection in pooled saliva samples: An effective cost-saving method

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Abstract

Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a causative agent of coronavirus disease 2019 (COVID-19), which was first reported in December 2019 and has since spread globally. Effective laboratory testing is crucial for the early detection and prevention of SARS-CoV-2 trans mission. The use of pooled saliva samples represents a potential method for active case finding and increasing testing efficiency.

Objectives: To evaluate the effectiveness of pooled saliva samples for SARS-CoV-2 testing and its cost-effectiveness for screening healthcare workers at King Chulalongkorn Memorial Hospital, Thai Red Cross Society.

Methods*:* A total of 24,098 samples collected between April 19, 2021, and May 30, 2022, to be tested for the presence of SARS-CoV-2 were analyzed retrospectively. The samples were examined individually and in pools of four and six using the Cobas 6,800 reverse-transcription polymerase chain reaction assay for the detection of SARS-CoV-2 RNAs. The analysis focused on changes in cycle threshold values for each target between positive pools and positive individual samples.

Results: SARS-CoV-2 was detected in 0.5% of the samples (123/24,098). Pooling saliva samples in groups of four or six did not compromise the detection of viral RNAs. Pooled saliva testing showed high performance for SARS-CoV-2 detection, with cost reductions of 73.5% for the four-sample pools and 80.7% for the six-sample pools compared with individual testing.

Conclusion: Pooling saliva samples is a cost-effective and efficient method for screening SARS-CoV-2, particularly in low-prevalence settings. This approach helps quickly identify and isolate healthcare workers with infection, thus reducing transmission and preserving resources.

Keywords: COVID-19, pooling saliva samples, RT-PCR, SARS-CoV-2.

The World Health Organization declared coronavirus disease (COVID-19) a pandemic on March 11, 2020, caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). [1] This declaration prompted nations to implement public health measures to manage disease transmission effectively. In December 2020, Thailand experienced another COVID-19 outbreak, in which many infected individuals exhibited mild symptoms or were asymptomatic despite having higher levels of the virus in their samples. [2] This highlights the potential for rapid transmission among asymptomatic individuals and underscores the importance of active case finding as a critical strategy for early disease detection. [3]

Saliva samples are a convenient, self-collected, and reliable alternative to nasopharyngeal (NP) swabs for detecting SARS-CoV-2. Saliva samples can be compared with NP swabs in real-time polymerase chain reaction (RT-PCR) assay for SARS-CoV-2 detection, with a reported sensitivity of 84.0%–100.0% and specificity of 89.0%–100.0%. [4-7] Pooled-sample COVID-19 testing has been proposed as a cost-effective and noninvasive method for larger-scale testing, particularly in a screening environment. [8] Testing SARS-CoV-2 RNA in pooled saliva samples produced accurate results, demonstrating a sensitivity of 98.0% when employing a pool size of five with heat pretreatment of saliva and 89% when utilizing

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the same pool size without heat inactivation. [9] Watkins AE, *et al.* also revealed that pooled saliva testing experienced a sensitivity decrease of 7.4%, 11.1%, and 14.8% for pool sizes comprising 5, 10, and 20 samples, respectively.[10]

This study presents saliva pooling as a method for active case finding to identify SARS-CoV-2 infection among healthcare workers at King Chulalongkorn Memorial Hospital, Thai Red Cross Society. Accordingly, this study aimed to compare the cycle threshold (Ct) value obtained for each pool with the individual positive sample and assess its cost-effectiveness.

Materials and methods

Study population

This study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University (IRB no. 0101/66, COA no. 0359/2023). The retrospective descriptive study was conducted with 24,098 saliva samples from the healthcare workers employed at King Chulalongkorn Memorial Hospital between April 19, 2021, and May 30, 2022. The pooling system was performed in two cohorts: in cohort A, each pool comprised four saliva samples (2,219 pools), and in cohort B, each pool comprised six saliva samples (2,537 pools). Healthcare workers are classified as high-risk individuals given the nature of their work, which entails direct interaction with many patients or those with suspected infection.

Specimen collection and processing

From each individual, saliva samples were collected by spitting 2–3 mL of saliva into sterile containers without any transport media. Subsequently, these samples were stored on ice and transported to the Department of Microbiology within 24 h before SARS-CoV2 RNA analysis. In cases where the sample included mucus, 500 µL of the saliva sample was initially treated by adding 10 µL of proteinase K (20 mg/mL, Invitrogen, Thermo Fisher Scientific, Waltham, MA), followed by vortexing, heating for 5 min at 56°C, and brief centrifugation. For pooling samples, the four-sample pools required a minimum of 200 µL of saliva from each sample (total of 800 µL), whereas the six-sample pools required at least 150 µL of saliva from each sample (total of 900 µL). A pooled saliva was added into a secondary tube, requiring a minimum volume of 600 µL for SARS-CoV-2 testing and then loaded onto an automated Cobas 6,800 instrument (Roche Molecular Diagnostics, Pleasanton, CA).

The testing process included sample extraction, PCR amplification, and SARS-CoV-2 detection utilizing a two-target strategy for virus detection. Target 1 focused on the *ORF1ab* nonstructural region, whereas target 2 aims the envelope (*E*) gene in all SARS-like coronavirus. [11] The following detection criteria were established: samples with both positive *ORF1ab* and *E* were considered "detected", whereas those with both negative genes were classified as "not detected". Inconclusive results, presumed positive for SARS-CoV-2 RNA, were defined by a negative result for *ORF1ab* and positive for *E* (**Table 1).**

In the case of positive results, each sample within the pool further underwent individual assessment to identify individuals with infection. The mean Ct value difference between the pooled and individual saliva samples was compared. [12]

Statistical analysis

The Wilcoxon signed-rank test was used to compare the median Ct value with 95% confidence intervals (CIs) between the pooled and individual saliva samples. A two tailed $P < 0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism version 10 (GraphPad Software, San Diego, CA)

Results

Four-sample pool

From April 19 to June 30, 2021, 8,876 saliva specimens were analyzed, organized into 2,219 pools, each comprising four samples. Among these, 2,185 pools yielded a "not detected" result. Positive cases were identified in 32 pools, detecting both *ORF1ab* and *E*, whereas two pools detected only *E*, resulting in an "inconclusive" result. Further analysis of the positive pools unveiled that 24 pools contained a single positive sample, six pools had two positive samples, and two pools exhibited three positive samples. In the individual assessment of the saliva samples, 43 samples had positive results, and one had an inconclusive result **(Table 2).**

Within the four-sample pool, the median Ct values for *ORF1ab* in the pooled and individual samples were 29.2 (22.3–34.8) and 28.8 (21.0–35.0), respectively. The median Ct values of *E* in the pooled and individual

samples were 30.1 (22.3–37.5) and 29.6 (20.9–36.4), respectively. In pools containing a single positive specimen, the comparison of the Ct value of *ORF1ab* in the four-sample pools with that obtained from the individual samples revealed a significant difference ($P = 0.0004$). The median change in Ct was +0.9 units (95% CI, 0.4–1.6). Similarly, when examining Ct values of *E* in the four-sample pools and individual specimens, a significant difference was observed ($P < 0.0001$), with a median change of +1.2 units (95% CI, 0.5–1.8) (**Figure 1).**

Table 1. Interpretation of the SAR-CoV2 testing by Cobas 6800 real time RT-PCR.

Target 1 (*ORF1ab* gene)	**Target 2 (*E* gene)**	**Interpretation**
Positive (Ct)	Positive (Ct)	Detected
Negative	Positive (Ct)	Inconclusive
Negative	Negative	Not detected

Table 2. Summary of the SARS-CoV-2 gene detection results of pools of four and six saliva samples.

	Positive number of pools		
	Total	***ORF1ab* and *E* genes**	***E* gene**
Pools of 4 samples	**34**	**32**	**2**
Positive 1 in 4	26	24	2
Positive 2 in 4	6	6	0
Positive 3 in 4	2	2	0
Pools of 6 samples	**67**	**55**	**12**
Positive 1 in 6	59	47	12
Positive 2 in 6	5	5	0
Positive 3 in 6	2	2	0
Positive 4 in 6	1	1	0

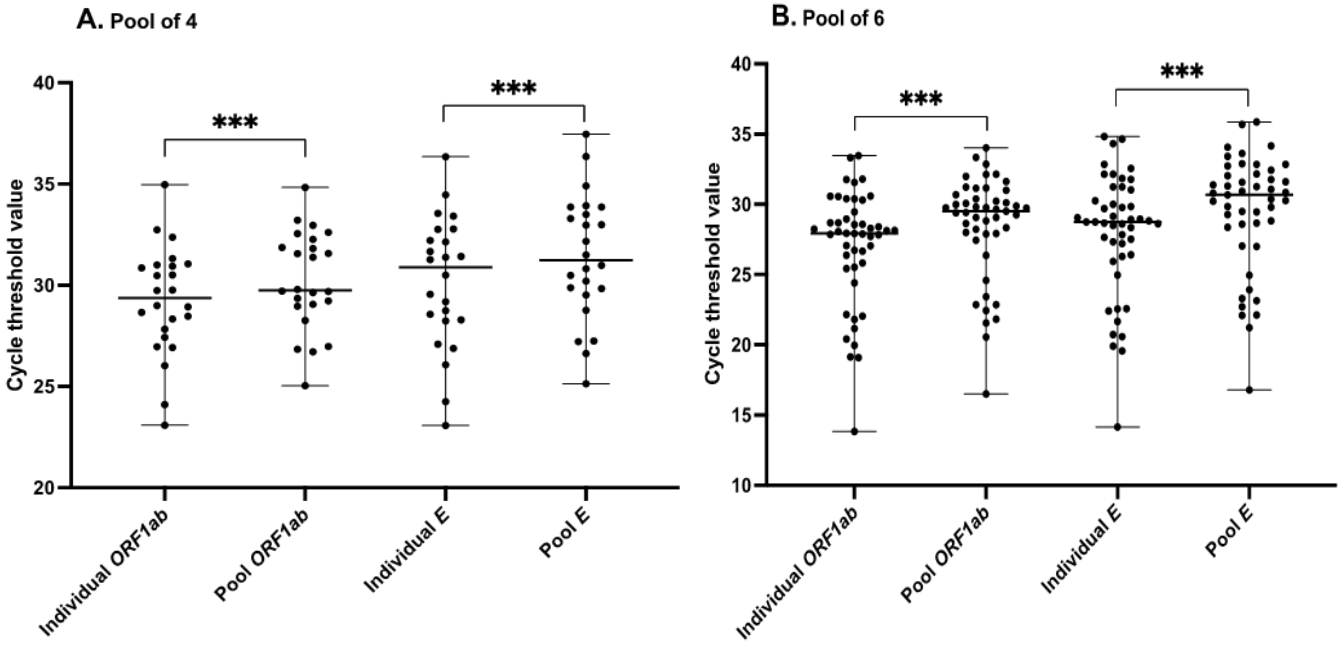

Figure 1. Comparison of the single positive Ct values of individual and pooled samples in the *ORF1ab* and *E* genes using 4-pool samples **(A)** and 6-pool samples **(B).** Wilcoxon determined statistical differences matched pairs signed rank test [$P < 0.05$ indicated by asterisks (***)]

The Ct values of both *ORF1ab* and *E* in pools containing two and three positive specimens were compared with the Ct values of individual specimens. The median change in Ct for *ORF1ab* ($P = 0.2462$) was –0.5 units (95% CI, –4.2 to 1.4), and for *E* ($P = 0.1964$), the median change in Ct was –0.5 units (95% CI, –4.2 to 1.4) (**Figure 2).**

Six-sample pools

From July 1, 2021, to May 30, 2022, 15,222 saliva specimens were analyzed, organized into 2,537 pools of six samples each. Among these pools, 2,470 showed "not detected" results, 55 were positive for both *ORF1ab* and *E*, and 12 only had *E*. The individual results of these positive pools led to the identification of 77 positive individual saliva samples and two inconclusive samples. Within the 55 positive pools, 47

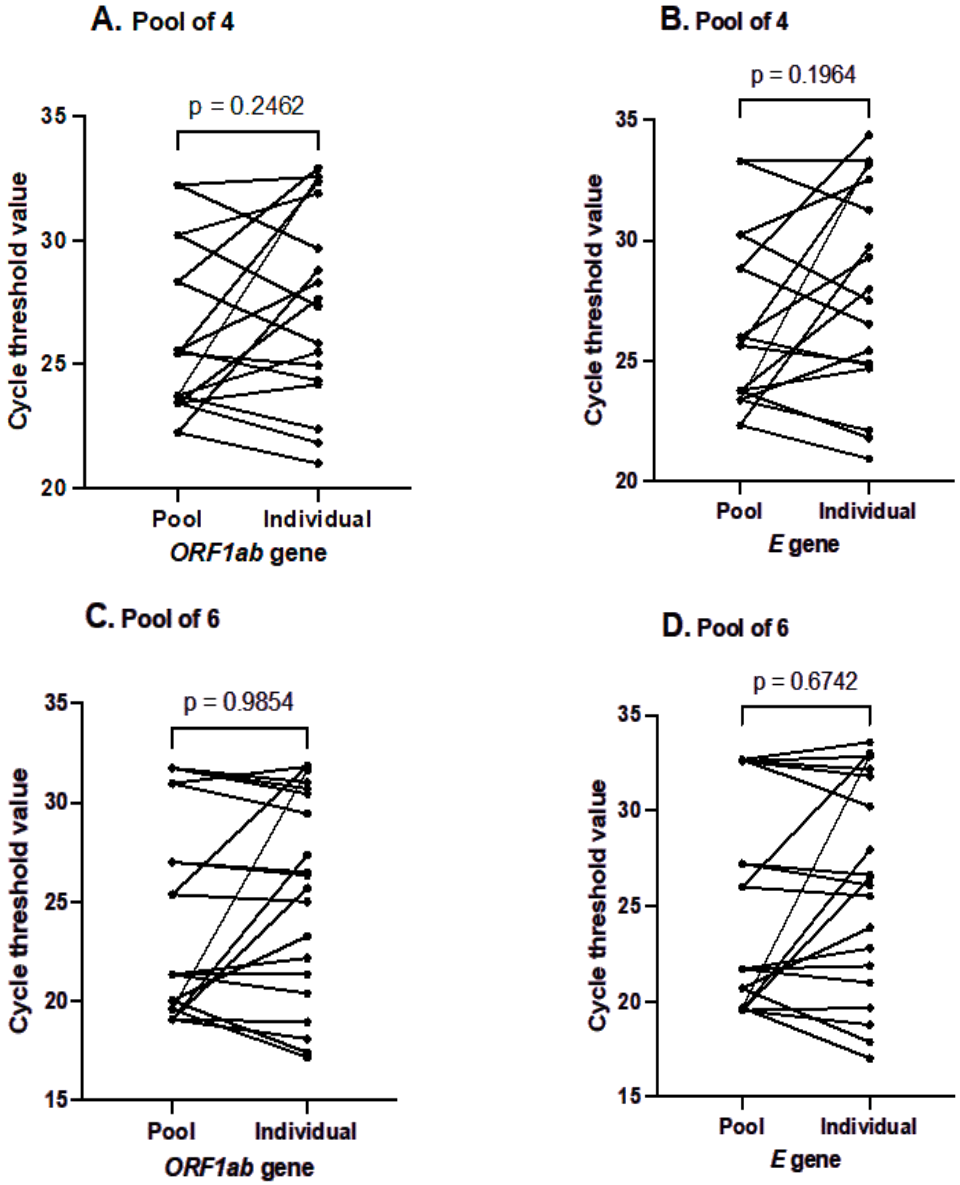

Figure 2. Cycle threshold values of *ORF1ab* and *E* genes for paired pools and individual samples containing more than one positive sample. Pairs are connected by a line: **(A)** and **(B)** are 4-pool sample;, and **(C)** and **(D)** are 6-pool samples. Wilcoxon determined statistical differences matched pairs signed-rank test ($P < 0.05$).

contained one positive sample, 5 contained two positive samples, 2 had three positive samples, and 1 included four positive samples. Within the six-sample pools, the median Ct values for *ORF1ab* in the pooled and individual samples were 29.1 (16.5–34.0) and 28.1 (13.8–33.5), respectively. The median Ct values for *E* in the pooled and individual samples were 28.2 (13.8–37.4) and 28.9 (14.2–36.4), respectively. A comparison of the Ct values for *ORF1ab* between the pooled and individual specimens revealed a highly significant difference ($P < 0.0001$), with a median change of +1.5 units (95% CI, 1.3–1.7). A similar trend was observed for *E*, where the Ct values in the six-sample pools versus individual specimens demonstrated a significant difference ($P < 0.0001$), with a median change of +1.9 units (95% CI, 1.5–2.1) (**Figure 1).**

Further analysis was conducted for pools with two, three, and four positive samples. The Ct values of both *ORF1ab* and *E* were compared with those of individual samples. The median change in the Ct values for *ORF1ab* $P = 0.9854$) and *E* ($P = 0.6742$) were +0.4 units (95% CI, –0.9 to 1.0) and +0.2 units (95% CI, –1.1 to 0.8), respectively (**Figure 2).**

Saliva pools with inconclusive results

Fourteen saliva pools were positive for only *E*. Eleven saliva samples were positive for both *ORF1ab* and *E* when analyzed individually. When the Ct value of *E* in the pools was compared with that of the individual samples, a significant difference ($P = 0.009$) was found. The median change in the Ct value was +2.0 units (95% CI, 0.5–3.0). The results for the remaining three samples were still inconclusive or were presumptive positive. The Ct values of *E* of the pools were 34.4, 36.9, and 36.3; however, the results for the individual samples were 35.0, 36.4, and 34.2, respectively **(Table 3).**

In this study, 4,655 negative and 101 positive pools were identified, detecting 123 positive saliva samples, with 120 showing positive results and three displaying inconclusive results, yielding a positivity rate of 0.5%. This successful identification enabled the isolation of individuals with infection, effectively restricting the spread of the virus throughout the community. Furthermore, implementing this protocol resulted in a significant cost reduction; specifically, the use of four-sample pools resulted in a 73.5% reduction in reactions, whereas the six-sample pools achieved an even more substantial decrease at 80.7% (**Table 4).**

Table 3. Inconclusive results of pools saliva samples.

Pooled result		Individual result	
ORF1ab	***E***	***ORF1ab***	***E***
Negative	35.5	33.2	34.2
Negative	34.4	Negative	35.0
Negative	35.5	32.1	33.9
Negative	37.4	32.5	34.5
Negative	35.4	30.3	33.1
Negative	36.9	Negative	36.4
Negative	36.4	Negative	34.2
Negative	35.5	32.0	34.8
Negative	37.0	32.3	34.3
Negative	37.0	31.7	33.8
Negative	36.8	33.0	34.9
Negative	37.4	31.9	34.1
Negative	35.2	32.7	35.0
Negative	35.9	31.3	33.6

Table 4. Estimation of SARS-CoV-2 Cobas 6800 cost comparing between pooling and individual sample analysis.

Pool size	Total samples	Total pools	No. of samples pool tested			Reaction use	Save reaction
			Negative	Positive	Inconclusive		
4	8,876	2,219	2,185	32	2	2,355	6,521 (73.5%)
6	15,222	2,537	2,470	55	12	2,939	12,283 (80.7%)

Discussion

The evaluation of the Roche Cobas 6800 RT-PCR assay for SARS-CoV-2 RNA using a six-sample pool demonstrated a sensitivity of 90.0%, which increased to 100.0% for samples with Ct values < 34. [13] In this study, the medians and ranges of the Ct values were 28.3 (13.8–35.0) for *ORF1ab* and 29.2 (14.2–36.4) for *E*. Another study found that pooled saliva testing can effectively detect a Ct shift, with a pool size of five without heat inactivation, resulting in an increased Ct value shift of 2.0, showing 89.0% positive agreement compared with individual testing. [9] Dilution may occur in pools with only one positive sample, potentially increasing the risk of false-negative results. [14] In this study, a total of 71 pools have only one positive sample, a significant Ct value shift of 1.4 in *ORF1ab* and 1.7 in *E*. However, some samples had potentially inconclusive results, attributed to factors such as low concentrations, proximity to or below the test's limit of detection, mutations in the *ORF1ab* target region, or presence of Sarbecoviruses not previously observed in humans. [15, 16] These factors significantly affect the performance of molecular diagnostic assays, raising concerns about false negatives for *ORF1ab* employed in rRT-PCR tests. [17] Mutations in the viral genome can contribute to the discrepancies in *ORF1ab* detection, potentially causing delays in detecting *ORF1ab* compared with other target genes, such as *E*. [16, 18]

The study revealed a COVID-19 prevalence of 0.5% among healthcare workers in King Chulalongkorn Memorial Hospital, Thai Red Cross Society, identifying 123 positive cases among 24,098 individuals screened for SARS-CoV-2 RNAs using RT-PCR between April 19, 2021, and May 30, 2022. The prevalence of COVID-19 among healthcare workers in Thailand from May 2020 to May 2021 was approximately 4.2%. [19] The variation in prevalence was attributed to the specified outbreak period. Implementing this pooling strategy can enhance laboratory testing capacity and reduce testing expenses in future outbreaks. The recommended sample pool size is 4–6 samples, previously validated in multiple studies that have revealed high agreement rates between pooled and individual testing. [20 - 23] This successful identification facilitated the isolation of positive individuals, effectively limiting virus spread in the community.

In addition, the pooling protocol demonstrated significant resource conservation. The findings underscore the cost-effectiveness of using pooled saliva samples for testing, particularly in populations with a low positivity rate (<1.0%), where assay costs can be reduced by approximately 80.0%. [24] In this study, pooled saliva testing reduced costs by 78.0%, making routine SARS-CoV-2 surveillance affordable and efficient for healthcare workers at King Chulalongkorn Memorial Hospital, Thai Red Cross Society.

However, the sensitivity of pooled testing was only 90.0%, [13] leading to potential false-negative results due to the dilution effects and inconclusive results caused by low viral concentrations or mutations. Future studies should focus on improving sensitivity, addressing dilution issues, adapting assays to detect viral mutations, and exploring new technologies to improve the efficacy and reliability of pooled testing.

Conclusion

This study demonstrated the efficiency of saliva pooling for detecting SARS CoV 2 RNAs. The use of pooled samples has increased the testing capacity, saved resources, and improved resource efficiency in low-resource environments. The optimal pool size depends on the specific context and infection prevalence. While pooling saliva samples can increase the testing throughput, the potential for false-negative results and the effect of the viral load on detection must be considered when implementing pooling strategies for testing, ensuring test reliability, and contributing to more effective and sustainable pandemic management approaches.

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Conflict of interest statement

All authors have completed and submitted the International Committee of Medical Journal Editors Uniform Disclosure Form for Potential Conflicts of Interest. None of the authors disclose any conflict of interest.

Data sharing statement

All data generated or analyzed in the present study are included in this published article. Further details are available for noncommercial purposes from the corresponding author on reasonable request.

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