



Effectiveness of a Sample Pooling Strategy to Detect SARS-CoV-2, Thimphu, Bhutan, January–February 2021

Sonam Gyeltshen*, Kunzang Dorji, Tshering Dorji, Vishal Chhetri

Royal Centre for Disease Control, Ministry of Health, Bhutan

*Corresponding author, email address: sonamgyeltshen@health.gov.bt

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Abstract

This retrospective study aimed to evaluate the effectiveness of a pooling strategy to detect SARS-CoV-2 by comparing cycle threshold (C_t) values of pooled samples and deconvoluted samples, and its cost savings. We evaluated 2,687 pools of 5 samples ($n=13,435$) and 5,444 pools of 10 samples ($n=54,440$). Of these, 51 and 40 pools were positive from the 5- and 10-sample pools, respectively. We analyzed the C_t values of positive samples obtained through pooled testing and compared them to the C_t values of their corresponding individual positive samples. We observed a decrease in the mean C_t value by 2.0 for the 5-sample pools and by 3.6 for the 10-sample pools when the pooled samples were deconvoluted and individually tested. The drop in the mean C_t value was more pronounced in samples with a low viral load. Where the known positive COVID-19 prevalence rate was 0.13%, the pooling strategy resulted in significant savings of 89.26% for pools of 10 samples and 78.10% for pools of 5 samples when compared to individual testing. Employing this pooling approach led to substantial savings in resources and time when conducting mass screening.

Keywords: COVID-19, SARS-CoV-2, pooling, cycle threshold, RT-PCR

Introduction

The detection of the first severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infected case on 5 Mar 2020 marked the beginning of Bhutan's comprehensive response to the coronavirus disease 2019 (COVID-19) pandemic.¹ COVID-19 testing was instrumental in Bhutan's response as part of the Test, Trace, and Treat (3T) approach for early identification of cases and to limit the transmission potential of the virus.²

Bhutan sealed its international borders to incoming travelers on 22 Mar 2020, all land and air routes were closed for non-emergency travel.¹ Thereafter, all individuals entering Bhutan via road and air were tested on days 3–5, days 12–14, and day 21 of quarantine prior to their release.¹ Routine testing was also initiated for individuals traveling within the country from high-risk to low-risk areas during a mandatory quarantine period of seven days. Frontline workers and a representative sample from high-risk populations were tested weekly and every two weeks, respectively. Furthermore, the Ministry of Health undertook mass screenings using the reverse transcription polymerase

chain reaction (RT-PCR) technique during the phase of local transmission. Consequently, the use of COVID-19 test kits increased, which resulted in a shortage of consumables and reagents.

This sample pooling strategy was initially used to detect HIV and the hepatitis B/C virus and during the H1N1 pandemic.^{3–5} The introduction of this strategy led to uninterrupted testing without requiring more laboratory personnel and/or test kits, improving efficiency, but also conserving resources.⁶ On 11 Aug 2020, the Royal Government of Bhutan imposed its first nationwide lockdown in response to the escalating community transmission of COVID-19.⁷ To mitigate the spread of the virus, the Ministry of Health undertook extensive testing efforts. Consequently, the demand for RT-PCR test kits surged. A sample pooling strategy was then introduced to curb the shortage of test kits, especially for COVID-19 screening during the mass testing.

Nonetheless, the impact of the pooling strategy on the cycle threshold (C_t) of individual positive tests and samples with a low viral load remained unclear.

Therefore, this paper aimed to determine the effectiveness of implementing a sample pooling approach in Bhutan during the COVID-19 pandemic.

Methods

Study Design

This study is a retrospective analysis using a sample pooling strategy implemented for COVID-19 testing at the Royal Centre for Disease Control using RT-PCR during the second nationwide lockdown in the capital city, Thimphu, between 2 Jan 2021 and 2 Feb 2021. A pool size of 5 was used for samples collected from primary contacts and quarantine facilities, while a pool size of 10 was used for samples collected from community screenings. The tests were performed using RT-PCR targeting the envelope (E) and ORF1ab genes. Positive pools were deconvoluted, and individual samples were tested for both the E and ORF1ab genes for confirmation. The C_t values of both pooled samples and individual samples were extracted from the PCR worksheet form.

Sample Collection

Nasopharyngeal swabs were collected from individuals in the community and their primary contacts, quarantine facilities, and travelers. Samples were stored in viral transport media and shipped to the Royal Centre for Disease Control for testing. Samples were processed within 24 hours from the time of their receipt.

Pooling Strategy, RNA Extraction and RT-PCR

RT-PCR was employed on two sets of pooled samples. The first group included 2,687 pools, each consisting of 5 samples, while the second group consisted of 5,444 pools with 10 samples each. Fifty μ L was collected from each pool of the 5-sample pools and 25 μ L from each pool of the 10-sample pools. These pooled samples were then transferred into 1.5 mL Eppendorf tubes. From each pooled sample, a 200 μ L aliquot was used for RNA extraction. The RNA extraction was performed using the STANDARD M SPIN-X viral extraction kit (SD BIOSENSER, South Korea) following the manufacturer's guidelines. Ten μ L of eluted RNA was subjected to RT-PCR testing using the Standard M nCoV Real-Time-Detection kit (SD BIOSENSER, South Korea), targeting the E and ORF1ab genes following the manufacturer's guidelines. Samples that tested negative in the pool testing were reported as negative, while those that tested positive were retested individually before the report was finalized.

Pooling as a Resource-saving Strategy

The cost-saving strategy was evaluated by comparing the total expenditure made for pooling strategies

(5-sample pools and 10-sample pools) against the total expenditure that would have been required for individual testing, considering a testing cost of US\$ 40 using Ngultrum 3000 per test during this period.

Pooling as a Time-Saving Strategy

During the study period, 18 laboratory staff were deployed: six for the RNA extraction, two for reagent preparation, two for sample sorting and labeling, and eight for sample pooling and aliquoting. The time required by 18 laboratory staff to process 94 individual samples per plates was approximately 15 minutes for sample processing, 40 minutes for RNA extraction, and an average run time of 1 hour and 30 minutes per PCR plate. However, the sample processing time for pools of 5 and 10 samples increased approximately by 45 and 85 minutes respectively, mainly due to pool preparation. The total time required for RNA extraction and the average PCR run remained the same. The time-saving strategy and testing efficiency were calculated by comparing the time required to test each plate of individual specimens (94 specimens per plate) processed with three PCR instruments per run, with the time spent for processing pools of 5 and 10 samples.

Data Analysis

Data were entered in Microsoft Excel. Descriptive statistics were used to analyze the results, and all analyses were done using Stata 13.1.

Ethics

This study was approved by the Research Ethical Board of Bhutan, Ministry of Health (REBH/Approval/2023/028).

Results

A total of 2,687 pools ($n=13,435$ samples) of 5-samples and 5,444 pools ($n=54,440$ samples) of 10-samples were evaluated in this study. Of these, 51 pools were positive from the 5-sample pools (1.89%) and 40 were positive from the 10-sample pools (0.73%). Pool-positive samples were deconvoluted and tested individually; 255 individual tests were performed from 51 pools of 5 samples and 51 (20%) individual samples were positive for SARS-CoV-2. Among the 10-sample pools, 40 pools (400 samples) were positive and among these samples, 40 (10%) were positive. The overall prevalence of the disease in laboratory setting was determined to be 0.13% (91/67,875). The C_t value of positive pools of the 10-sample pools for the E gene assay ranged from 17.3 to 42.0, and 19.25 to 40.36 for the ORF1ab gene. The C_t value of positive pools for the 5-sample pools for the E gene assay ranged from 15.5 to 42.34 and 16.92 to 40.20 for ORF1ab gene (Table 1).

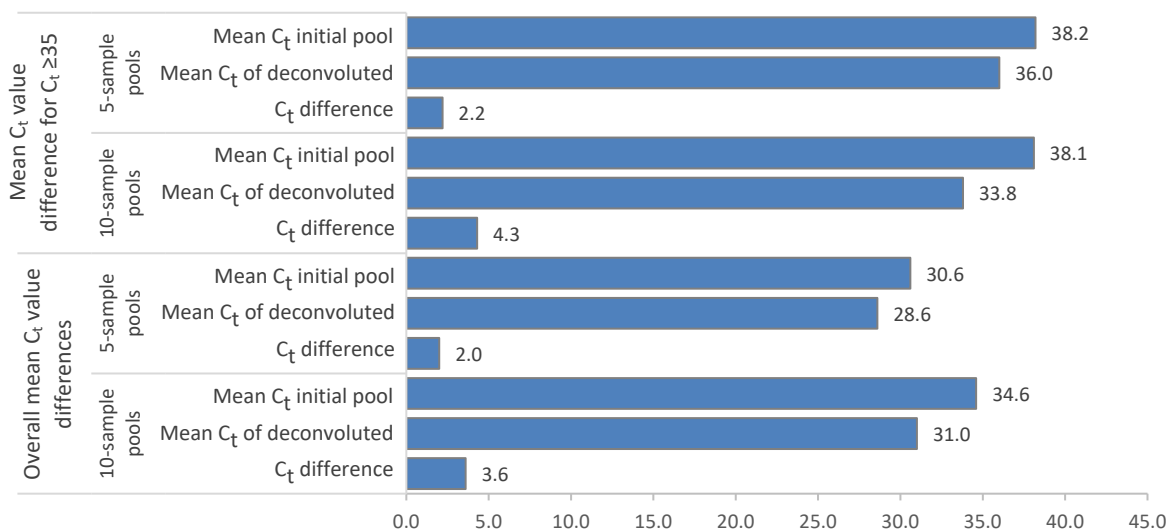
Table 1. Comparison of pooled data between 10- and 5-sample pools, Thimphu, Bhutan, January to February 2021

Gene tested	Pool and deconvoluted data	Minimum C_t value	Maximum C_t value	Mean C_t value $\pm$ SD
E gene	• 5 sample pools (n=51)	15.50	42.34	31.01 $\pm$ 8.4
	• Deconvoluted samples from 5 sample pools (n=255)	15.16	40.01	28.73 $\pm$ 8.1
	• 10 sample pools (n = 40)	17.30	42.00	34.96 $\pm$ 7.1
	• Deconvoluted samples from 10 sample pools (n = 400)	15.05	38.27	31.16 $\pm$ 6.7
ORF1ab gene	• 5 sample pools (n=51)	16.92	40.20	30.23 $\pm$ 7.4
	• Deconvoluted samples from 5 sample pools (n=255)	15.12	39.08	28.42 $\pm$ 7.4
	• 10 sample pools (n = 40)	19.25	40.36	34.30 $\pm$ 5.7
	• Deconvoluted samples from 10 sample pools (n = 400)	15.01	36.89	30.75 $\pm$ 5.4

C_t : cycle threshold. SD: standard deviation.

On average, when comparing the differences in C_t values between pooled samples and deconvoluted samples, the mean differences in C_t values for the pools of 5 and 10 samples were 2.0 and 3.6, respectively.

When we specifically analyzed the mean C_t value differences in positive pooled samples with a C_t value ≥ 35 , these differences became more pronounced. (Figure 1).

**Figure 1. Comparison of overall mean cycle threshold (C_t) value differences and mean differences for $C_t \geq 35$, Thimphu, Bhutan, January to February 2021**

Conducting individual tests for all 67,875 samples would have resulted in a cost of approximately US\$ 2,715,000. However, by implementing the sample pooling strategy, the cost was reduced to US\$ 233,760 (pools of 10 samples) and US\$ 117,680

(pools of 5 samples). The pooling approach was capable of reducing costs, by 89.26% for a pool of 10 samples and 78.10% for a pool of 5 samples, when compared to conducting individual tests (Table 2).

Table 2. Cost comparison for specimen pooling using reverse transcription polymerase chain reaction, Thimphu, Bhutan, January to February 2021

	10-sample pools	5-sample pools
Total samples	54,440	13,435
Number of pools tested	5,444	2,687
Number of pools positive	40	51
Number of individual tests required	400	255
Total number of tests required	5,844	2,942
Number of positive tests detected	40	51
Cost per test*	40	40
Cost without sample pooling strategy*	2,177,600	537,400
Cost with sample pooling strategy*	233,760	117,680
Total cost savings (%)	89.26	78.10

*All costs are in US dollars.

Testing a batch of 94 individual samples per plate in a single run required a total time of 145 minutes. Utilizing the pooling approach, we tested 470 samples per plate (in pools of 5) and 940 samples per plate (in pools of 10) within 175 minutes and 215 minutes, respectively in a single run. Testing 13,435 samples individually would take 203.08 hours. However, with the pool of 5 samples, it took only 61.83 hours, including the testing of deconvoluted pooled positive samples. This represents a 3.28-fold improvement in testing efficiency. Similarly, testing 54,440 samples individually would have required 820.25 hours, but pooling them into 10-sample pools reduced the time to 160.66 hours, resulting in a 5.10-fold improvement in testing efficiency. Therefore, the pooling approach saved 69.55 of the time for a pool of 5 samples and 80.41 for a pool of 10 samples compared to individual testing.

Discussion

During the response to COVID-19 in 2021, the implementation of our sample pooling approach in a laboratory demonstrated significant advantages in enhancing SARS-CoV-2 testing capacity. Furthermore, our approach was simple and time-efficient, and effectively minimized resource consumption, including the use of reagents and testing materials.

We observed a drop in the mean C_t value of 2.0 for 5-sample pools and 3.6 for 10-sample pools. Our findings align with those of a 2020 study by Praharaj et al. that reported C_t value variances of 2.18 ± 1.86 cycles for 5-sample pools and 3.81 ± 2.26 cycles for 10-sample pools. A more recent study in 2022 by Rajamani et al documented C_t differences of 2.6 ± 0.67 cycles for 5-sample pools and 4.3 ± 1.47 cycles for 10-sample pools.^{8,9} Similar studies conducted in India, Spain, Japan, and the UAE also reported decreases in C_t values when implementing a pooling strategy.^{10–13} In a real-time RT-PCR test that operates with 100% amplification efficiency, one would anticipate an approximate difference of 3.3 C_t in a sample of 10-fold dilution.¹⁴ The disparities in average C_t values observed in our study between individual and pool positive were in line with this expectation.

We observed a slight increase in mean C_t value differences for both pools of 5 and 10 samples when analyzing cases with C_t values >35 . A similar study conducted in India highlighted the potential for missing individual positive cases when viral loads are very low.¹⁵ The drop in mean C_t values associated with low viral loads can be attributed to the fact that as the number of negative pooled samples increases, the amplified RNA takes longer to reach the threshold, which could be the expected outcome of sample dilution associated with the principle of the sample dilution

effect.¹⁶ Research from Egypt has emphasized that the pooling strategy can affect the test's sensitivity when dealing with samples exhibiting weak viral loads.¹⁶ Consequently, it is crucial to exercise caution when interpreting results from pooled samples. Any subtle indications or minor amplifications in a single gene should prompt individual testing to ensure that positive cases are not overlooked.

Our study demonstrated that employing a sample pooling method can enhance testing efficiency while concurrently reducing the resources required for SARS-CoV-2 testing. A positive rate of 0.13% resulted in savings of 89.26% for pools of 10 and 78.10% for pools of 5 samples. Similar studies have reported the cost-effectiveness of the pooling method.^{17–19} Sample pooling strategies also reduced the turnaround time for reporting.^{20,21} In our study, the testing efficiency of pools of 10 samples was more effective than pools of 5 samples, with the former increasing testing efficiency by 5.10-fold, and the latter by 3.28-fold. A similar study from India demonstrated that a 5-sample pool was more effective than a 10-sample pool with efficiency increases of 2.15-fold for the 5-sample pool and 1.8-fold for the 10-sample pool.²¹ The difference in the effectiveness of the pooling approach could be due to the higher prevalence rate in their setting. Our study demonstrated that implementation of a pooling approach saved 69.55% of the time for a pool of 5 samples and 80.41% for a pool of 10 samples. The implementation of a pooling strategy not only substantially reduced resource consumption but also significantly decreased processing time, allowing for efficient management of the overwhelming demand for testing.

Limitations

A limitation of this study was the lack of generalizability as it was carried out within a single laboratory, confined to a specific geographical region, and focused solely on assessing two distinct pool sizes. Additionally, the lack of published data on the prevalence rate of SARS-CoV-2 infection in the study area during the study period further limited the analysis. Furthermore, the study was conducted in a real-life setting, preventing the determination of sensitivity and specificity for the pooling strategy, as there were no instances of negative pool samples being deconvoluted and retested.

Conclusion

In summary, considering the benefits and drawbacks of adopting the pooling technique, especially in a low-resource environment, pooling samples proved more advantageous and effective. The pooling technique helped expand the testing capacity and addressed the challenges associated with mass testing while also

lowering costs, reducing the required workforce, and saving time. However, prior to implementing the pooling strategy, it is essential for all laboratories to perform validation studies specific to their testing kits and extraction/amplification platforms, considering the known COVID-19 prevalence rate.

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Conflicts of Interests

No conflict of interest declared.

Suggested Citation

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