Evaluating a Modified Sputum Preparation Method for Improved qRT-PCR Detection of COVID-19

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Abstract

Background: An appropriate specimen is the crucial for quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) detection of COVID-19. Sputum is suitable specimen due to its noninvasive and simple collection process. For reference method, sputum samples were prepared by adding normal saline in a 1:2 ratio, followed by mixing and vortexing until fully dissolved. However, its sticky and vicious nature can hinder the preparation process and delay results. Therefore, a modified sample preparation was required during the COVID-19 pandemic.

Objective: To compare a modified sample preparation method with reference method for sputum specimens in the detection of COVID-19 by qRT-PCR.

Methods: Leftover sputum specimens were included and prepared in parallel using both the modified sample preparation and reference methods. qRT-PCR for COVID-19 was performed using the Seegene Allplex SAR-CoV2 master assay. Sensitivity, specificity, and other performance parameters were statistically analyzed. Cycle threshold (Ct) values of target genes (E, RdRp/S, N, and S variants) from both methods were compared.

Results: A total of 202 samples were examined. The modified sample preparation and reference methods yield positive results of 32 (15.8%) and 31 (15.3%), inconclusive results of 1 (0.5%) and 5 (2.5%), invalid results of 4 (2.0%) and 27 (13.4%), and negative results of 165 (81.7%) and 139 (68.8%), respectively. The sensitivity and specificity were 86.7% and 100%, respectively, with positive predictive value of 100% and negative predictive value of 97.1%. There were no statistically significant differences in Ct values for the E, RdRp/S, N, and S variants between the 2 methods with *P* values of .169, .421, .513, and .172, respectively.

Conclusions: The modified sample preparation method can be used during a COVID-19 pandemic while maintaining the reliable accuracy and reducing turnaround time.

Keywords: qRT-PCR, Sputum sample, Modified sample preparation method, COVID-19

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Introduction

SARS-CoV-2 causes a severe respiratory infectious disease known as COVID-19, leading to a worldwide outbreak with human-to-human transmission. COVID-19 was first reported as a deadly pneumonia in Wuhan, Hubei Province, China. It became a massive burden worldwide, and the World Health Organization (WHO) declared it a pandemic in March 2020. SARS-CoV-2 affected an upper respiratory system, causing flu-like symptoms such as cough, sore throat, and fever which can lead to respiratory failure, pneumonia, and even fatality. SARS-CoV-2 is closely associated to SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), which shares 80% and 50% in sequence identity, respectively.[1]

SARS-CoV-2 is an enveloped, positive- sense-single-stranded RNA virus, with a 30-kb genome and belongs to the *Coronaviridae* family. Its genome encodes the structural glycoproteins, including spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins.[1, 2] There are several diagnostic approaches for classifying COVID-19, which include clinical suspicion, epidemiological data, and diagnostic testing. Since, the clinical symptoms resemble other respiratory infection diseases, the epidemiological data are important alongside with laboratory diagnosis. Nucleic acid tests, particularly quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR), is considered as the gold standard for early COVID-19 detection.[3-5]

A qRT-PCR detection of SARS-CoV-2 is recommended for early diagnosis, with nasopharyngeal sampling presumed to have the highest diagnostic yield.[6] However, alternative samples such as nasal swabs, oropharyngeal swabs, throat swabs, oropharyngeal swabs, tracheal aspiration, and sputum are also used for qRT-PCR detection.[7-13] The viral load in each of sample types can vary depending on the infection period.[7] Among these samples, sputum appears to have the highest detectability when compared to other specimens.[10, 12]

At Khon Kaen Hospital, a significant number of sputum samples were collected for COVID-19 detection by qRT-PCR. Pretreatment of sputum is necessary before nucleic acid extraction. As sputum's viscous nature can make sample preparation time-consuming. There are several methods to pretreat sputum before a nucleic acid extraction, such as diluting with molecular-grade water in a 1:1 ratio followed by vortexing for 15 to 30 minutes,[14] or using the nonionic detergent Triton X-100 and the endonuclease alcalase to disrupt mucus in sputum.[15] Diluting sample with normal saline in a 1:2 ratio, which was recommended by the qRT-PCR reagent vendor, was timely. A simpler and faster sputum pretreatment method is needed to reduce turnaround time and ensure consistent accuracy. This study thus aimed to compare COVID-19 detection using sputum pretreated with normal saline and a modified sample preparation method where sputum is placed into a viral transport medium before nucleic extraction.

Methods

Sample Collection and Sample Preparation Methods

This study was conducted at Khon Kaen Hospital between October and December 2022. Using sputum samples submitted for COVID-19 testing. Parallel preparations of sputum were performed using 2 methods, a reference method and a modified sample preparation method, followed by qRT-PCR detection. In the reference method, sputum samples were dissolved in normal saline at a 1:2 ratio (sputum: normal saline) and homogenously mixed. In the modified method, a portion of each sputum sample was placed in viral transport media using a flocked swab.

Viral Nucleic Extraction and qRT-PCR

A volume of 200 µL of the prepared sample was parallel extracted by using a nucleic acid extraction kit and the EXM3000 extraction system (Zybio Inc, Germany). The eluted RNAs were detected by qRT-PCR by using the Allplex SARS-CoV2 Master Assay (Seegene Inc, Republic of Korea) according to the manufacturer's instructions. Cycle threshold (Ct) of target genes and housekeeping gene were recorded.

Interpretation of qRT-PCR for COVID-19

The results were interpreted according to the COVID-19 interpretation's protocol of the Department of Medical Sciences, Ministry of Public Health, Thailand.[16] The Ct values of the target genes including E, RdRp/S, N, and S variants genes, were compared. A valid internal quality control, indicated by housekeeping gene, required a Ct value of less than 40. A 'detectable' result means that 2 of all target genes were detected result (Ct < 40). An 'undetectable' result means that none of the target genes were detected (Ct > 40). An 'inconclusive' result means only one target genes was detected; in such case, testing should be repeated or the specimen should be recollected. In this study, only 'detectable' results were included for comparison between the 2 methods. An 'invalid' result, defined as, internal controls with a Ct value of greater than 40 or no Ct value, were excluded.

Statistical Analysis

The detectable and undetectable results from the modified sample preparation method were included to calculate the performance parameters, including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy, were calculated using a contingency table. Samples that yielded invalid or inconclusive results in one or both methods were excluded from statistical calculation. For comparisons, the Wilcoxon signed rank test was performed using SPSS version 27.0 (IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp; 2020), with a $P < .05$ considered statistically significant. Ct values of all detectable samples were plotted as a scatter plot using GraphPad Prism version 9.0.0 (GraphPad Software).

Results

qRT-PCR Detection Result for COVID-19

A total of 202 sputum samples were prepared in parallel using 2 methods; collecting with a flocked swab into a viral transport medium and dissolving with normal saline prior to nucleic acid extraction, followed by the detection of qRT-PCR for COVID-19. The data demonstrated that the modified sample preparation method and reference method showed detectable results in 32 (15.8%) and 31 (15.3%) samples, respectively. Inconclusive results were found in 1 (0.5%) and 5 (2.5%) samples, invalid results in 4 (2.0%) and 27 (13.4%) samples, and undetectable results in 165 (81.7%) and 139 (68.8%) samples, respectively (Table 1).

Table 1. Comparison of Quantitative Reverse Transcriptase-Polymerase Chain Reaction for COVID-19 Results Between Modified and Reference Methods

Result	No. (%)	
	Modified Method	Reference Method
Detectable	32 (15.8)	31 (15.3)
Inconclusive	1 (0.5)	5 (2.5)
Invalid	4 (2.0)	27 (13.4)
Undetectable	165 (81.7)	139 (68.8)
Total	202 (100.0)	202 (100.0)

The 165 samples with undetectable and detectable results from the modified sample preparation method were used to calculate sensitivity and specificity compared to the reference sample preparation method. The data showed that sensitivity and specificity were 86.7% and 100%, respectively. The PPV, NPV, and accuracy were 100%, 97.1%, and 97.6%, respectively (Table 2).

Comparative Statistical Analysis of Ct Values for COVID-19 Target Genes Between Modified and Reference Sample Preparation Methods

This study found no significant differences of Ct values of E, RdRp/S, N, and S variants between the 2 methods (Table 3). The mean of Ct values were calculated for a total of 33 COVID-19 detectable samples, including the Ct value of inconclusive results by the modified and normal saline methods, while invalid results were excluded. For the modified sample preparation method, the mean (95% CI) of Ct values were 27.41 (23.40-30.83), 29.09 (23.96-31.79), 25.90 (22.89-30.89), and 28.45 (24.50-33.96) for E, RdRp/S, N, and S variants genes respectively. Meanwhile, the mean (95% CI) of Ct values for the normal saline method were 22.42 (19.55-31.22), 24.76 (20.40-32.34), 25.29 (20.44-34.29), and 24.40 (20.96-32.10) for E, RdRp/S, N, and S variants genes, respectively (Figure 1).

Table 2. Performance Parameters of the Modified and Reference Methods

Modified Method	No. of Sample			Performance Parameters of the Modified Method, %				
	Reference Method							
	Detectable	Undetectable	Total	Sensitivity	Specificity	NPV	PPV	Accuracy
Detectable	26	0	26	86.7	100.00	100.00	97.1	97.6
Undetectable	4	135	139					
Total	30	135	165					

Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

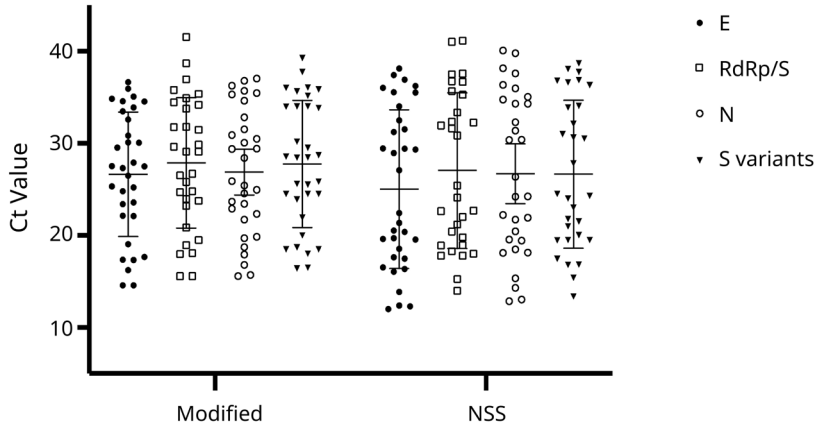
Table 3. Statistical Analysis Comparing Between a Modified Method and a Reference Method

Gene	Z Score*	*P* Value
E	1.376**	.169
RdRp/S	-0.804**	.421
N	-0.654**	.513
S	-1.365**	.172

* Wilcoxon signed rank test.

** Based on positive ranks.

Figure 1. Comparison of Cycle Threshold (Ct) Values (Mean [95% CI]) of Target Genes Between a Modified Method and a Reference Method



Modified = Modified method, NSS = Reference method

Discussion

Early and precise diagnosis for COVID-19 is the key to effective epidemic prevention and control. The appropriate specimen and the infection period are crucial for accurate detection. For early diagnosis, a nasopharyngeal swab is recommended and has been shown to have the highest diagnostic yield compared to other specimens.[6] However, sputum is an alternative specimen for COVID-19 nucleic acid detection, especially in patients with lower respiratory symptoms, such as pneumonia. Sputum collection is noninvasive, simpler and provides a high diagnostic yield. Nevertheless, its sticky and viscous nature make preparation time-consuming and difficult.

In this study, we modified the sputum preparation process to simplify and faster in order to reduce turnaround time and minimize the factors that may lead to an invalid result. Practically, the modified sample preparation was suitable during COVID-19 pandemic, given the high number of sputum specimens delivered for COVID-19 detection by qRT-PCR. In contrast, the reference method was time-consuming and resulted in more invalid samples. The sputum preparation had to be repeated in the reference method, leading to waste of reagents and other supplies. This study found that the modified method yielded a higher proportion of valid results compared to the reference method. The sticky and viscous nature of sputum makes it difficult to dissolve effectively with normal saline. Thus, the modified sample preparation method is more practical and simpler than the reference method.

Factors such as a preparation technique, sample appearance, sample volume might affect accuracy. Inconclusive and invalid results were excluded from sensitivity and specificity calculations. As a result, the modified sample preparation method achieved a sensitivity of 86.7% and a specificity of 100%. It is also important to note that the ideal specimen for COVID-19 detection vary with the infection duration.[17] A systemic review found that sputum specimen is better than nasopharyngeal swab or oropharyngeal swab,[18] consistent with the studies by Zang et al[19] and Whang et al.[20] In contrast, Chan et al[21]

and Liu et al[22] did not find any significant differences in positivity between sputum, nasopharyngeal swab, and oropharyngeal swab. Sharma et al[17] noted that sputum collection might not be suitable for the patients of COVID-19 infection with dry cough who cannot produce sputum.

This study further compared the Ct values of target genes between the 2 preparation methods. To reduce interference, the invalid results were excluded from the comparative statistical analysis. This study found that the Ct values of the reference sample preparation method were slightly lower (indicating higher yield) than those from the modified sample preparation method. However, there was no significant difference between the modified and reference sample preparation methods ($P > .05$). Therefore, the modified sample preparation method can be used as an alternative to the reference method for COVID-19 detection by qRT-PCR during the COVID-19 pandemic. This is the first report of this modification, which has now been implemented as a routine method in our molecular laboratory, resulting in reduced turnaround time while maintaining accuracy.

Conclusions

The modified sputum preparation method showed high sensitivity, specificity, and accuracy, making it a suitable alternative to the time-consuming reference method.

Additional Information

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Ethics Approval: This research was approved by the Human Research Ethics Committee of Khon Kaen Hospital, Thailand (KEXP65054 on 5 September 2022).

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Formal Analysis: All authors
Investigation: All authors
Methodology: All authors
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References

1. Naqvi AAT, Fatima K, Mohammad T, et al. Insights into SARS-CoV-2 genome, structure, evolution, pathogenesis and therapies: structural genomics approach. *Biochim Biophys Acta Mol Basis Dis.* 2020;1866(10):165878. doi:10.1016/j.bbadis.2020.165878
2. Sawicki SG, Sawicki DL, Siddell SG. A contemporary view of coronavirus transcription. *J Virol.* 2007; 81(1):20-29. doi:10.1128/jvi.01358-06

3. Caliendo AM, Hanson KE. *COVID-19: Diagnosis.* UpToDate. 18 December 2024. Accessed 24 December 2024. https://www.uptodate.com/contents/covid-19-diagnosis
4. Zhou P, Yang XL, Wang XG, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature.* 2020;579(7798):270-273. doi:10.1038/s41586-020-2012-7
5. Coronaviridae Study Group of the International Committee on Taxonomy of Viruses. The species *Severe acute respiratory syndrome-related coronavirus*: classifying 2019-nCoV and naming it SARS-CoV-2. Nat Microbiol. 2020;5(4):536-544. doi:10.1038/s41564-020-0695-z
6. US Food and Drug Administration. FAQs on Testing for SARS-CoV-2. 11 August 2023. Accessed 24 December 2024. https://www.fda.gov/medical-devices/coronavirus-covid-19-and-medical-devices/faqs-testing-sars-cov-2
7. Vandenberg O, Martiny D, Rochas O, van Belkum A, Kozlakidis Z. Considerations for diagnostic COVID-19 tests. *Nat Rev Microbiol.* 2021;19(3):171-183. doi:10.1038/s41579-020-00461-z
8. Lin C, Xiang J, Yan M, Li H, Huang S, Shen C. Comparison of throat swabs and sputum specimens for viral nucleic acid detection in 52 cases of novel coronavirus (SARS-Cov-2)-infected pneumonia (COVID-19). *Clin Chem Lab Med.* 2020;58(7):1089-1094. doi:10.1515/cclm-2020-0187
9. Akowuah E, Acheampong G, Ayisi-Boateng NK, et al. Comparable detection of SARS-CoV-2 in sputum and oropharyngeal swab samples of suspected COVID-19 patients. *COVID.* 2022;2(7):858-866. doi:10.3390/covid2070062
10. Uddin MKM, Shirin T, Hossain ME, et al. Diagnostic performance of self-collected saliva versus nasopharyngeal swab for the molecular detection of SARS-CoV-2 in the clinical setting. *Microbiol Spectr.* 2021;9(3):e0046821. doi:10.1128/spectrum.00468-21
11. Tsujimoto Y, Terada J, Kimura M, et al. Diagnostic accuracy of nasopharyngeal swab, nasal swab and saliva swab samples for the detection of SARS-CoV-2 using RT-PCR. *Infect Dis.* 2021;53(8):581-589. doi:10.1080/23744235.2021.1903550
12. Fan G, Qin X, Streblow DN, Hoyos CM, Hansel DE. Comparison of SARS-CoV-2 PCR-based detection using saliva or nasopharyngeal swab specimens in asymptomatic populations. *Microbiol Spectr.* 2021;9(1):e0006221. doi:10.1128/spectrum.00062-21
13. Kiryanov SA, Levina TA, Kadochnikova VV, Konopleva MV, Suslov AP, Trofimov DY. Clinical evaluation of nasopharyngeal, oropharyngeal, nasal swabs, and saliva for the detection of SARS-CoV-2 by direct RT-PCR. *Diagnostics.* 2022;12(5):1091. doi:10.3390/diagnostics12051091
14. Thwe PM, Ren P. Analysis of sputum/tracheal aspirate and nasopharyngeal samples for SARS-CoV-2 detection by laboratory-developed test and panther fusion system. *Diagn Microbiol Infect Dis.* 2021;99(1):115228. doi:10.1016/j.diagmicrobio.2020.115228
15. Guzman NA, Guzman DE. A COVID-19 infection with positive sputum and negative nasopharyngeal/oropharyngeal rapid antigen-based testing: a case report and pilot study. *Med Clin Case Rep J.* 2023;1(2):77-84. doi:10.51219/mccrj/norberto-a-guzman/22
16. Department of Disease Control, Ministry of Public Health. Guidelines for Surveillance and Investigation of Coronavirus Disease 2019 (COVID-19). Published 1 December 2021. Updated 22 December 2021. Accessed 24 December 2024. https://ddc.moph.go.th/viralpneumonia/eng/file/guidelines/g_GSI_22Dec21.pdf
17. Sharma K, Aggarwala P, Gandhi D, et al. Comparative analysis of various clinical specimens in detection of SARS-CoV-2 using rRT-PCR in new and follow up cases of COVID-19 infection: quest for the best choice. *PLoS One.* 2021;16(4):e0249408. doi:10.1371/journal.pone.0249408
18. Mohammadi A, Esmaeilzadeh E, Li Y, Bosch RJ, Li JZ. SARS-CoV-2 detection in different respiratory sites: a systematic review and meta-analysis. *EBioMedicine.* 2020;59:102903. doi:10.1016/j.ebiom.2020.102903
19. Zhang H, Chen M, Zhang Y, et al. The yield and consistency of the detection of SARS-CoV-2 in multiple respiratory specimens. *Open Forum Infect Dis.* 2020;7(10):ofaa379. doi:10.1093/ofid/ofaa379

20. Wang W, Xu Y, Gao R, et al. Detection of SARS-CoV-2 in different types of clinical specimens. *JAMA.* 2020;323(18):1843-1844. doi:10.1001/jama.2020.3786
21. Chan JF, Yuan S, Kok KH, et al. A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster. *Lancet.* 2020;395(10223):514-523. doi:10.1016/S0140-6736(20)30154-9
22. Liu R, Han H, Liu F, et al. Positive rate of RT-PCR detection of SARS-CoV-2 infection in 4880 cases from one hospital in Wuhan, China, from Jan to Feb 2020. *Clin Chim Acta.* 2020;505:172-175. doi:10.1016/j.cca.2020.03.009