Tropical Biomedicine 41(1): 64-69 (2024)
https://doi.org/10.47665/tb.41.1.008

RESEARCH ARTICLE

A colorimetric reverse transcription-loop mediated isothermal amplification (RT-LAMP) method for the rapid detection of SARS-CoV-2 in Thailand

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ARTICLE HISTORY

Received: 14 July 2023
Revised: 13 September 2023
Accepted: 13 September 2023
Published: 25 March 2024

ABSTRACT

COVID-19, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), remains a global health threat. Timely identification of infected cases is important for appropriate patient management and the control of viral spread. Simple and cost-effective tests are required to increase access to testing and early case detection. Here, we describe a colorimetric reverse transcription-loop-mediated isothermal amplification (RT-LAMP) method to detect SARS-CoV-2. The RT-LAMP could amplify the *orf1ab* sequence detectable by visual color change within 45 min at 63 °C. The limit of detection (LoD) for SARS-CoV-2 RNA was less than 100 copies (13.36) per reaction with no cross-amplification with other related viruses. Clinical evaluation using leftover RNA samples extracted from 163 nasopharyngeal swab specimens showed perfect agreement in negative (n = 124) and positive samples with cycle thresholds (Ct) < 34 cycles (n = 33) detected by real-time reverse transcription-polymerase chain reaction (RT-PCR), targeting *RdRp* and *N* genes as a reference. Overall, the diagnostic accuracy, sensitivity, specificity, positive and negative predictive values of RT-LAMP in testing were 96.32% (95% CI: 92.16-98.64%), 84.62% (95% CI: 68.47-94.14%), 100% (95% CI: 97.07-100.0%), 100% (95% CI: 89.42-100.0%), and 95.38% (95% CI: 90.22-98.29), respectively. This RT-LAMP assay is simple and reliable, with the potential to be an alternative for the rapid detection of SAR-CoV-2 with minimal time and fewer resources compared to real-time RT-PCR.

Keywords: SARS-CoV-2; COVID-19; RT-LAMP; RT-PCR; validation.

INTRODUCTION

The coronavirus disease 2019 (COVID-19), a respiratory infection caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (Zhu *et al*., 2020), is an emerging global health threat that has generated continuous global health problems over three years. The disease has infected numerous people worldwide with more than 768 million cases and 6.9 million deaths as of 17 June 2023 (WHO, 2023). The viral surges in multiple outbreaks have been reported to correspond to low immunity, inadequate protective responses, or the emergence of new variants (Hacisuleyman *et al*., 2021; Mistry *et al*., 2022). In Thailand, COVID-19 was declared an endemic disease in October 2022. Since then, some daily cases and deaths have occurred from time to time, leading to public concerns in certain areas.

Rapid and reliable identification of SARS-CoV-2 infection is one of the key strategies for the prevention and control of the disease. Different methods for SARS-CoV-2 detection have been developed and employed (Li *et al*., 2020; Safiabadi Tali *et al*., 2021). Among these, the real-time reverse transcription-polymerase chain reaction (RT-PCR) assay is the most reliable and considered a reference standard for COVID-19 diagnostics (Corman *et al*., 2020). Although real-time RT-PCR testing has high sensitivity and specificity, the technique requires complex facilities, costly equipment, and reagents, including skilled laboratory personnel. In addition, it requires a long operation time of at least 2–4 hours with numerous liquid handling, rendering it unsuitable for large-scale testing or rapid detection, especially in low-resource settings (Feng *et al*., 2020; Mannier & Yoon, 2022). Simple, reliable, and cost-effective tests that can identify infected persons quickly are important for decelerating the spread of SARS-CoV-2.

The loop-mediated isothermal amplification (LAMP) is a molecular test with great potential as a point-of-care tool since it requires minimum resources, less preparation, and a short turnaround time. The method offers an alternative to PCR by nucleic acid amplification under isothermal conditions (Notomi *et al*., 2000). The reactions comprise a set of 4–6 primers targeting 6–8 template regions and a strand displacement polymerase to amplify specific DNA quickly (Nagamine *et al*., 2002; Notomi *et al*., 2000). LAMP assays can be carried out using simple incubators, such as a small

heating block or water bath, with readouts by gel electrophoresis, turbidity, fluorescence intensity, or visual color changes (Tomita *et al*., 2008; Wong *et al*., 2018).

The reverse transcription-LAMP (RT-LAMP) combines the transcription step with isothermal amplification to detect viral RNA (Huang *et al*., 2020; Ali *et al*., 2022). The reverse transcription and LAMP reactions could be run together in a single tube to shorten the reaction time (Huang *et al*., 2020; Jiang *et al*., 2020). Colorimetric RT-LAMP assays for the rapid detection of SARS-CoV-2 RNA have been developed through naked-eye visualization of color changes in the reactions (Lamb *et al*., 2020; Choi *et al*., 2023). The methods are versatile and can be broadly adjusted using various approaches (Lamb *et al*., 2020; Aldossary *et al*., 2022; Ali *et al*., 2022). RT-LAMP assays have advantages compared to rapid antigen tests. They are relatively easy to operate, provide fast results, and offer higher sensitivity and specificity (Peeling *et al*., 2022, Hossain *et al*., 2022). Although each test has some limitations, RT-LAMP testing is simple, fast, and cost-effective, with high sensitivity and specificity comparable to real-time RT-PCR (Amaral *et al*., 2021; Ali *et al*., 2022). In addition, RT-LAMP assays are useful since they can extend the range of available test methods. In this study, the RT-LAMP assay was assembled as an alternative for the rapid detection of SARS-CoV-2 in the Thai population. The RT-LAMP methodology and the validation of its performance in comparison to the gold standard real-time RT-PCR are described in the following section.

MATERIALS AND METHODS

Study design and samples

The study was designed to establish an alternative colorimetric RT-LAMP and evaluate its analytical and clinical performance for the detection of SARS-CoV-2. The detection results were compared to those of real-time RT-PCR using remnant RNA samples. The study protocol was approved by the Ethics Committee of the Development of Human Research Protection**,** Ministry of Public Health (MoPH), Thailand.

RT-LAMP primers

The primer set targeting *orf1ab*, as designed by Lamb *et al.* (2020), was used. The sequences of each oligonucleotide primer are listed in Table 1. The 10X primer mix was prepared as primer stock concentrations, which included an outer forward primer (F3), outer backward primer (B3), forward inner primer (FIP), backward inner primer (BIP), loop forward primer (LF), and loop backward primer (LB), and the final assay concentrations of each primer were 0.2 µM F3/B3, 1.6 µM FIP/BIP, and 0.4 µM LF/LB.

Colorimetric RT-LAMP

Reaction mixtures were prepared at room temperature immediately before testing using WarmStart® Colorimetric LAMP 2X Master Mix (New England Biolabs, Ipswich, MA, USA). The assay was set up in a total volume of 25 µl according to the manufacturer's instructions. A final reaction contained 1X WarmStart® Colorimetric LAMP Master Mix, 5 µl of nuclease-free water, 2.5 µl of 10X primer mix in the concentrations described earlier, and each 5 µl RNA sample was added immediately before starting the reaction. The reactions were then incubated at 63 °C for 45 min (60–65 °C and 30–60 min were tested) on a thermocycler (Axygen® MaxyGeneII Thermal Cycler, Corning, NY, USA) or heating block and then removed to stop the reaction on ice. A positive result was determined by the naked eye visualizing a color change from pink to yellow. The color of the finished reactions was recorded using a mobile phone camera or an office scanner to document the color change. To optimize the incubated reactions, the temperature and time were varied from 60–65 °C for 30–60 min.

Table 1. RT-LAMP primer sequences used for SARS-CoV-2 detection in this study

Primers	Sequences (5' to 3')
F3	TCCAGATGAGGATGAAGAAGA
B3	AGTCTGAACAACTGGTGTAAG
FIP	AGAGCAGCAGAAGTGGCACAGGTGATTGTGAAGAAGAAGAG
BIP	TCAACCTGAAGAAGAGCAAGAACTGATTGTCCTCACTGCC
LF	CTCATATTGAGTTGATGGCTCA
LB	ACAAACTGTTGGT CAACAAGAC

For positive control, SARS-CoV-2 standard RNA was prepared from a stock concentration of 534,576.42 RNA copies number/ml at dilutions ranging from 1:1,000–1:10,000. This positive standard was available from a real-time RT-PCR test kit employed in this study. The negative control was nuclease-free water with no positive SARS-CoV-2-RNA template. Positive and negative controls were included in all running assays.

Determination of analytical specificity in the RT-LAMP

The specificity of this set of primers was analyzed extensively and reported previously (Lamb *et al*., 2020). In this study, only five RNA samples were tested as a reference from related viruses other than SARS-CoV-2, namely Influenza AH1 and B (Flu A H1, Flu B), Respiratory Syncytial Virus A and B (RSV A, B), and Middle East Respiratory Syndrome (MERS) to further validate the specificity of the assay. The RT-LAMP reaction products were also analyzed using a 2% agarose gel.

Determining the analytical sensitivity of the RT-LAMP

The analytical sensitivity RT-LAMP assay was assessed based on the detection limits. The dilutions of known copy numbers per ml of RNA were prepared by diluting at 1:10, 1:100, 1:1,000, 1:200, 1:400, and 1:2,000 from a stock concentration of SARS-CoV-2 RNA (534,576.42 RNA copies number/ml) and used to determine the limit of detection (LoD) of the RT-LAMP assay. Samples were added to the RT-LAMP reactions and tested in triplicate at 63 °C for 45 min. Photographs were taken to record the pink-to-yellow color change in positive samples.

Clinical sample processing

Surplus RNA sample materials were used to evaluate the clinical sensitivity, specificity, and accuracy of the RT-LAMP. Nasopharyngeal swab (NPS) specimens were collected from individuals suspected of having COVID-19 as part of routine patient care and kept in a 2 ml viral transport medium (VTM). The collected samples were then transported in sterile containers at 4 °C, delivered to the laboratory, and tested by real-time RT-PCR at the diagnostic laboratory of the Regional Medical Sciences Center 11/1 Phuket, Department of Medical Sciences, MoPH, Thailand.

RNA extraction and real-time RT-PCR

The total RNA was extracted from 200 µl of VTM samples using a viral RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. From each sample, viral RNA was eluted with 30 µl of RNase-free water, and 2 µl and 5 µl were used as RNA templates for detecting SAR-CoV-2 by real-time RT-PCR and RT-LAMP assays, respectively.

A real-time RT-PCR analysis was conducted using DMSc COVID-19 Real-Time RT-PCR assay developed by the Division of Respiratory Virus Infection, Department of Medical Sciences, MoPH, Thailand (Okada *et al*., 2020). This development subsequently became a commercial quantitative RT-PCR product manufactured by the Siam Bioscience Co., Ltd. and used by many health authorities in Thailand. The assay was targeted at detecting the RNA-dependent RNA polymerase (*RdRp*) and Nucleocapsid (*N*) gene sequences. Samples considered positive for SARS-CoV-2 were either the detectable *RdRp* or *N* gene at a cycle threshold (Ct) < 40, according to the test kit instructions.

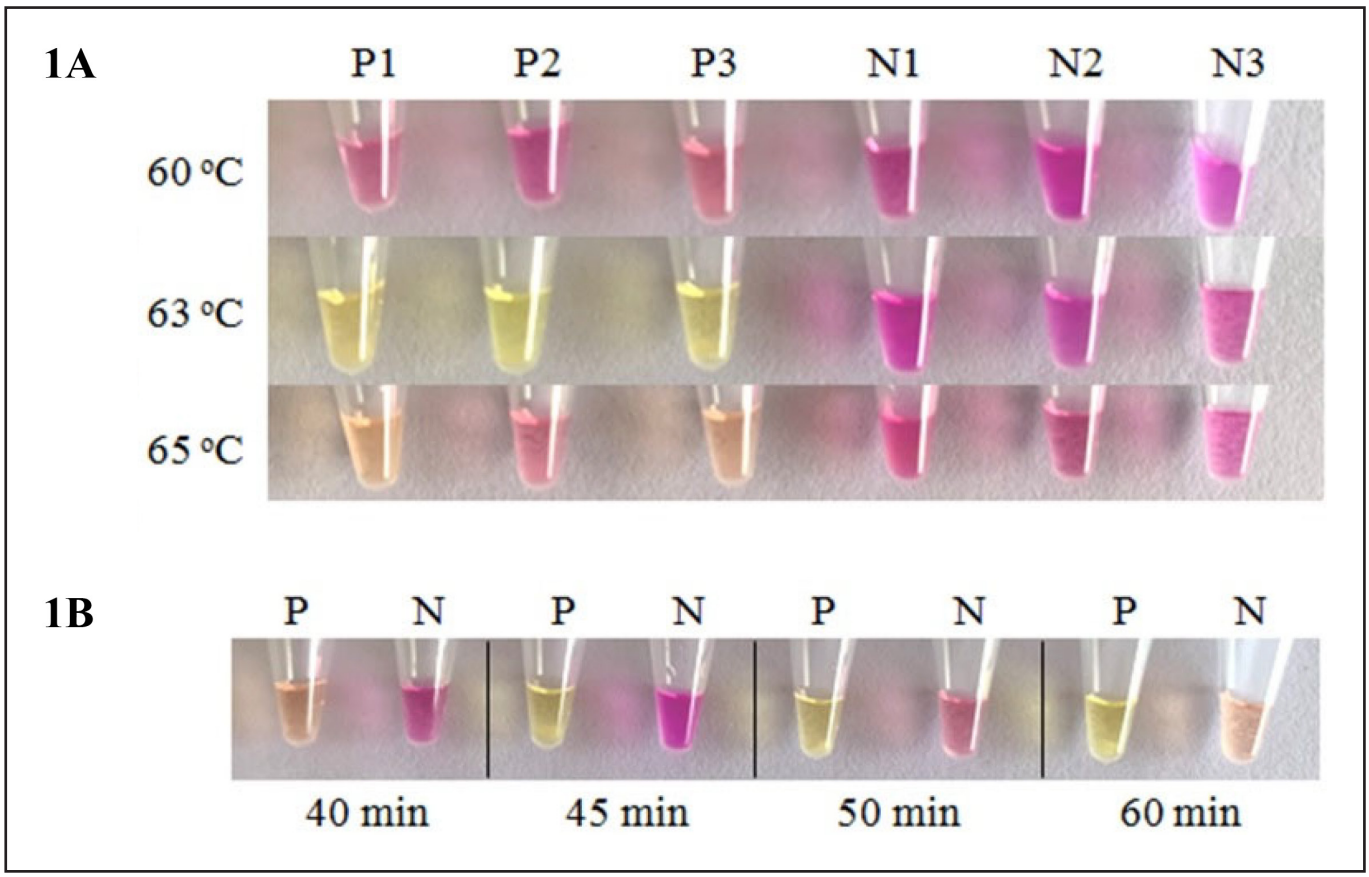

Figure 1. Determination of the optimal incubation temperature (1A) and time (1B) of the RT-LAMP reactions for the detection of SARS-CoV-2 viral RNA (P = Positive, N = Negative).

Clinical evaluation of the RT-LAMP

A leftover total RNA extracted from NPS specimens was used to evaluate the clinical performance of the developed assays. RT-LAMP testing was carried out as previously described. The reactions were incubated at 63 °C for 45 min in a heating block or on a conventional thermal cycler. Positive and negative controls were included in all running tests. Visual inspection of the color change in reaction samples was carried out to reveal the RT-LAMP results.

Data and statistics analysis

Descriptive data were presented in number or percentage formats with a 95% confidence interval (CI). The data from diagnostic test performance presented as sensitivity (the fraction of those with COVID-19 correctly identified), specificity (the fraction of those without COVID-19 correctly identified), positive predictive value (PPV), negative predictive value (NPV), and accuracy of the RT-LAMP assay were analyzed by comparing the results to those of the real-time RT-PCR testing as a reference and then calculated.

RESULTS

Optimized RT-LAMP

The RT-LAMP method using the WarmStart® Colorimetric LAMP Master Mix and a specific primer set was able to amplify SARS-CoV-2 viral RNA. The optimal incubation temperature and time for the RT-LAMP reactions could be established by testing a range of temperatures from 60–65 °C and times from 30–60 min. The optimized conditions for amplification were achieved at 63 °C for 45 min with the optimal concentrations of Master Mix, primers, RNA sample, and nuclease-free water. Figure 1 shows the determination results of the optimal incubation temperature and time of the RT-LAMP reactions.

Analytical specificity of the RT-LAMP

The specificity of RT-LAMP primers used in this study was tested for cross-reactivity with RNA from Flu AH1, Flu B, RSV A, RSV B, and MERS viruses. It was confirmed that this set of RT-LAMP primers retained specificity without any cross-reaction against tested viruses (Figure 2). Using agarose gel electrophoresis, the typical

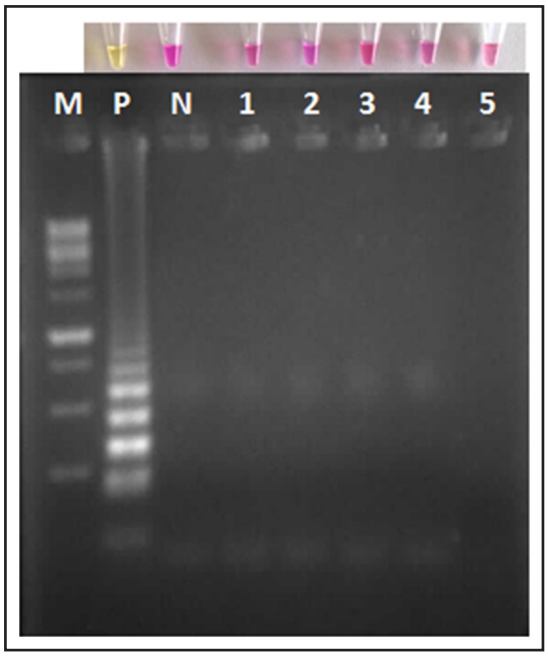

Figure 2. Determination of the specificity of the RT-LAMP assay for the detection of SARS-CoV-2 viral RNA by color visualization and agarose gel electrophoresis.

Table 2. The analytical sensitivity of the RT-LAMP assay for the detection of SARS-CoV-2 RNA

Sample	SAR-CoV-2 RNA Samples	Copies/mL	Copies/µL	Copies/reaction	Positive/Total tested
1	Undiluted	534,576.42	534.58	NA	NA
2	Diluted 1:10	53,457.64	53.46	267.30	3/3
3	Diluted 1:100	5,345.76	5.35	26.73	3/3
4	**Diluted 1:200**	**2,672.88**	**2.67**	**13.36**	**3/3**
5	Diluted 1:400	1,336.44	1.34	6.68	2/3
6	Diluted 1:1,000	534.58	0.53	2.67	2/3
7	Diluted 1:2,000	267.29	0.27	1.34	1/3

Table 3. Diagnostic performance of the RT-LAMP assay in comparison to real-time RT-PCR detection

		Ct (*RdRp*)	RT-LAMP		Accuracy (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
			Positive	Negative					
		≤ 25.00	17	0	100% (97.66-100%)	100% (89.11-100%)	100% (97.07-100%)	100% (89.11-100%)	100% (97.07-100%)
		25.01-30.00	10	0					
		30.01-35.00	5	0					
		35.01-40.00	1	6					
	Positive	**Ct (*N*)**							
rtRT-PCR		**≤ 25.00**	20	0	99.37% (96.52-99.98%)	97.06% (84.67-99.93%)	100% (97.07-100%)	100% (89.42-100%)	99.20% (95.62-99.98%)
		25.01-30.00	8	0					
		30.01-35.00	5	1					
		35.01-40.00	0	5					
		Total	**33**	**6**	**96.32% (92.16-98.64%)**	**84.62% (69.47-94.14%)**	**100% (97.07-100%)**	**100% (89.42-100%)**	**95.38% (90.22-98.29%)**
	Negative		**0**	**124**					

In comparison with *RdRp* detection by real-time RT-PCR, samples with Ct > 36.04 were undetectable by RT-LAMP.
In comparison with *N* detection by real-time RT-PCR, samples with Ct >34.67 were undetectable by RT-LAMP.
rtRT-PCR: real-time reverse transcription-polymerase chain reaction.

banding pattern for a positive RT-LAMP reaction was visible only in the sample containing SARS-CoV-2 RNA (positive control) while the corresponding color change from pink to yellow could be observed in this reaction within 45 min.

Analytical sensitivity of the RT-LAMP

The detection limit was assessed using a set of dilutions for known concentrations of SAR-CoV-2 viral RNA. The results from triplicate reactions indicated that the LoD of the RT-LAMP reaction was as low as 13.36 copies/reaction in 3/3 samples (less than 100 copies). The results for the RT-LAMP detection limit are summarized in Table 2.

Clinical validation of RT-LAMP detection

The leftover RNA samples extracted from 163 NPS specimens analyzed previously by real-time RT-PCR were obtained for analysis by the RT-LAMP assay. The Ct values of 39 real-time RT-PCR positive samples ranged from < 25 to 39.92 cycles with a mean value of 26.24 for the *RdRp* gene and from < 25 to 36.96 cycles with a mean value of 25.25 for the *N* gene (Tables 3 and 4). The distribution of Ct values among positive cases is summarized in Table 3. Approximately 82% of the positive samples had Ct values < 35 cycles. The RT-LAMP results could be compared to those of real-time RT-PCR based on the Ct values and the clinical performance of the RT-LAMP assay could be analyzed, as presented in Table 3.

Table 4. Discordant results detected by the RT-LAMP compared to real-time RT-PCR for SARS-CoV-2 detection

No.	RT-LAMP	Ct of RT-PCR		RT-PCR interpretation
		RdRp	*N*	
1	Negative	36.86	35.91	Positive
2	Negative	36.04	34.67	Positive*
3	Negative	39.92	37.43	Positive
4	Negative	38.77	36.96	Positive
5	Negative	38.15	36.33	Positive
6	Negative	37.57	36.95	Positive

*The Ct (s) of real-time RT-PCR for the detection of *RdRp* and *N* genes that could not be detected by RT-LAMP were ≥ 36.04 and 34.67, respectively (Sample 2).

The comparative detection analysis identified discordant results in six samples, all of which were negative for the RT-LAMP but positive for the real-time RT-PCR, as shown in Table 4.

According to the clinical evaluation, the RT-LAMP assay demonstrated potentially good performance for the detection of SARS-CoV-2. The comparative results showed 100% negative

agreement (n = 124) and provided concordance in all positive samples with Ct < 36.04 cycles (n = 33) for the real-time RT-PCR targeting the *RdRp* gene and < 34.67 cycles for the *N* gene, respectively (Tables 3 and 4). The accuracy, PPV, and NPV with CI values of the RT-LAMP are summarized in Table 3. Overall, the diagnostic accuracy, sensitivity, and specificity of the RT-LAMP in testing were 96.32% (95% CI: 92.16-98.64%), 84.62% (95% CI: 68.47-94.14%), and 100% (95% CI: 97.07-100%), respectively. The overall PPV and NPV were 100% (95% CI: 89.42-100%), and 95.38% (95% CI: 90.22-98.29%), respectively.

The clinical sensitivity was further calculated based on the Ct values of the real-time RT-PCR (Table 3). Of all the samples with Ct values ≤ 35, the RT-LAMP exhibited 100% and 97.06% sensitivity compared to the real-time RT-PCR targeting *RdRp* and *N* gene, respectively (Table 3). Based on the Ct values targeting both *RdRp* and *N* genes, the sensitivity of the RT-LAMP was 100% in samples with Ct values < 34.67 cycles (Tables 3 and 4). A decline in the positivity rate for samples with Ct values > 35 cycles was observed; the positivity rate decreased to 1/7 (14.29%) - 0/5 (0%) in samples with Ct values for *RdRp* and *N* genes > 35 cycles, respectively. According to the Ct cycles, the RT-LAMP exhibited 100% sensitivity for samples with Ct values of less than 34 cycles, dropping off rapidly above this level due to the decline in viral load shown in the tested samples.

DISCUSSION

Since the beginning of COVID-19 emergence, real-time RT-PCR assays have been extensively used while different diagnostic tests or methods have been developed or improved to identify infected cases with SARS-CoV-2; each may have some limitations. Rapid antigen tests are becoming increasingly important as a point-of-care device or a self-test at home for easier and faster SARS-CoV-2 detection. However, the major disadvantage of antigen tests is that they are less accurate than molecular detection (Peeling *et al*., 2022). The meta-analysis reported the pooled sensitivity and specificity of antigen tests as approximately 68.4% and 99.4%, respectively (Khandker *et al*., 2021). Since simple and cost-effective diagnostics are crucial for facilitating access to testing and increasing early case detection, a simple molecular test-based RT-LAMP assay has been described and proposed in this study as an alternative for the rapid detection of COVID-19. The assay was achieved by adding a set of specific primers and each RNA sample at optimal concentrations in an optimized RT-LAMP mixture using WarmStart® Colorimetric LAMP Master Mix at a final volume of 25 µl according to the manufacturer's instructions. The primer set as described was selected because it was reported that the RT-LAMP assays based on this target exhibited high sensitivity and specificity for SARS-CoV-2 detection (Lamb *et al*., 2020; Bhadra *et al*., 2021). In addition, the *orf1ab* which encodes non-structural proteins was reported to be highly conserved and specific to SARS-CoV-2 (Li *et al*., 2021). The method also combined a simple readout by observing the color change in the reactions from pink to yellow within a short time of 45 min, faster than the real-time RT-PCR methods. An overall sensitivity satisfaction of 84.62% and specificity values of 100% were obtained in a range similar to previous studies mostly reporting sensitivity values of 75–90% in several colorimetric RT-LAMPs for the detection of SARS-CoV-2 (Subali & Wiyono, 2021).

The clinical performance of this RT-LAMP method could be assessed using residual RNA from patient NPS samples with corresponding RT-PCR Ct values. For samples with Ct values ≤ 34 cycles as measured by a real-time RT-PCR reference, the RT-LAMP assay could detect SARS-CoV-2 at a rate of 100%. For samples with Ct values > 35 cycles, the RT-LAMP assay was much less sensitive. This suggested that a detection limit of the colorimetric RT-LAMP assay corresponded to Ct values equivalent to 34–35 cycles. The previous study proposed that the Ct values above 36 cycles correspond to less than 10 molecules or copies of RNA (Vogels *et al*., 2020). Our data on LoD at 13.36 copies per reaction and the detection limit around 34-35 cycles correspond to the estimation of the RNA copy number based on Ct values as described by Vogels *et al*. (2020).

This RT-LAMP was sensitive according to the LoD approximately as low as 13.36 viral copies. Lamb *et al*. (2020) reported the detection limit of their developed RT-LAMP assay at about 304 viral copies. Using the same set of primers could detect SAR-CoV-2 RNA as low as 100 copies reported by Bhadra *et al*. (2021). The sensitivity of the RT-LAMP in clinical detection could be varied test by test. In addition, the sensitivity could be affected by other factors such as specimen types and quality, collecting and processing, and the viral load in specimens. Varied sensitivity of RT-LAMP methods has been reported based on Ct values which represented viral RNA levels in the samples (Dao Thi *et al*., 2020). Besides, the sensitivity of the RT-LAMP assays might be varied according to the reference standard used in the comparison. Dao Thi *et al*. (2020) reported that about one-third of real-time RT-PCR positive samples had Ct values of 30–40 cycles. In this study, about 70% of samples had Ct values < 30 cycles, and around 18% of samples had Ct values > 35-40 cycles. Thus, the majority of COVID-19 cases > 80% should be detected by this RT-LAMP assay. Overall, the RT-LAMP was sensitive enough and could be a substitute for use in laboratories especially when the real-time RT-PCR is not feasible or in poor resource settings. Alternatively, the suspicious patients with an antigen-negative test result could be further examined rapidly by the RT-LAMP assay.

There are some limitations to this study. Surplus RNA sample materials were used from a routine diagnostic procedure rather than newly collected clinical samples. Therefore, the potential of the RT-LAMP method could not be evaluated for the direct detection of SARS-CoV-2 in unprocessed or crude samples. However, some studies have reported that RT-LAMP assays could directly detect SARS-CoV-2 in specimens without purification but achieved lower sensitivity (Dao Thi *et al*., 2020; Huang *et al*., 2020). In addition, the ability of the RT-LAMP assay to detect SARS-CoV-2 in a variety of specimens was not tested. The other limitation was the sample size. Despite the limitations in the study, good performance of the RT-LAMP method was demonstrated for use in detecting SARS-CoV-2.

CONCLUSION

This RT-LAMP assay demonstrated good performance for the rapid detection of SARS-CoV-2. The RT-LAMP in this study extends the variety of available test methods with fast, simple, and potentially cost-effective performance characteristics. This test is promising and can be used as an alternative for rapid case detection with minimal time and fewer resources compared to real-time RT-PCR assays.

ACKNOWLEDGEMENTS

This work was supported by the Department of Medical Sciences, MOPH, Thailand. The partial support by National Research Council of Thailand (NRCT grant N35A640044) to RJ was acknowledged. We thank the staff of the Regional Medical Sciences Center 11/1 Phuket, and the National Institute of Health, Department of Medical Sciences for their assistance and cooperation to do this research.

Conflict of interest

The authors declare that they have no conflict of interest.

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