JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Coronavirus Pandemic

An alternative colorimetric RT-LAMP assay for the rapid detection of SARS-CoV-2: development and validation in Thailand

Wiphat Klayut[1], Thanee Wongchai[2], Nattagarn Chuenchom[2], Payu Bhakdeenuan[1], Supranee Bunchoo[1], Sopa Srisungngam[1], Sirilada Suphankong[3], Benjawan Phetsuksiri[4], Ballang Uppapong[1], Janisara Rudeeaneksin[1]

[1] National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand
[2] Mae Sot Hospital, Ministry of Public Health, Tak, Thailand
[3] Regional Medical Sciences Center 5 Samut Songkhram, Department of Medical Sciences, Ministry of Public Health, Samut Songkhram, Thailand
[4] Medical Sciences Technical Office, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand

Abstract

Introduction: COVID-19, an emerging infectious disease caused by SARS-CoV-2, continues to be a global public health threat. The development of a colorimetric reverse transcription loop-mediated isothermal amplification (RT-LAMP) can extend the availability of simple, reliable molecular tests for the rapid detection of COVID-19.

Methodology: The RT-LAMP assay was developed using a new primer set targeting a portion of SARS-CoV-2 *orf8*. The method was validated at 63 ºC for 60 minutes with naked-eye visualization of the color change. The clinical performance was compared to a real-time reverse transcription-polymerase chain reaction (rtRT-PCR) using 273 RNA samples extracted from nasopharyngeal swab specimens.

Results: The developed RT-LAMP was specific to SARS-CoV-2 with a limit of detection at 15 RNA copies per reaction. The assay demonstrated diagnostic accuracy, sensitivity, specificity, positive predictive value, and negative predictive value of 90.48% (95% CI: 86.36–93.68%), 87.00% (95% CI: 81.53–91.33%), 100% (95% CI: 95.07–100%), 100% (95% CI: not available), and 73.74% (95% CI: 66.22–80.07%), respectively, compared to the rtRT-PCR. The greatest sensitivity of 98.03% (95% CI: 94.34–99.59%) was demonstrated in samples with the cycle threshold (Ct) values < 30 cycles.

Conclusions: The RT-LAMP method in this study showed good performance. The assay can increase the scope of laboratory testing for rapidly detecting SARS-CoV-2 in Thailand. Due to a decrease in COVID-19 cases, its application is beneficial when commercial alternatives are unavailable.

Key words: COVID-19; SARS-CoV-2; RT LAMP; rtRT-PCR; colorimetric.

J Infect Dev Ctries 2024; 18(8):1196-1203. doi:10.3855/jidc.19442

(Received 25 October 2023 – Accepted 30 November 2023)

Introduction

Coronavirus disease 2019, also known as COVID-19, an emerging respiratory infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1], remains a global health threat. For effective patient management and to control the spread of COVID-19, SARS-CoV-2 infection must be quickly and accurately detected. Simple and reliable diagnostics are important for improving access to testing and early case detection. Real-time reverse transcription-quantitative polymerase chain reaction (rtRT-PCR) is highly sensitive and specific, making it the gold standard for diagnosing COVID-19 [2,3]. Although RT-PCR is a fast method and can deliver results in a real-time manner, the testing takes hours to complete and requires well-trained personnel and well-equipped laboratories with expensive instruments like a real-time thermocycler as well as costly reagents. These factors limit its widespread application [4]. Currently, a variety of point-of-care tests for the timely detection of SARS-CoV-2 infection are readily available and often utilized [4–6]. Among these, lateral flow immunochromatography assays for detecting SARS-CoV-2 antigens are reasonably priced and simple to perform, even by self-testing for quick results in under 15 minutes. However, a significant problem with antigen testing for the early detection of COVID-19 is the reduced sensitivity compared to molecular detection [7]. According to a previous meta-analysis report, the

pool sensitivity and specificity of the antigen tests were about 68.4% and 99.4%, respectively [8].

Loop-mediated isothermal amplification (LAMP) has been proposed as a simple, fast, sensitive, specific, and inexpensive molecular test using nucleic acid amplification under isothermal conditions [9]. The reaction requires 4–6 oligo primers targeting 4–6 template regions and strand displacement polymerase to amplify specific DNA templates efficiently and rapidly at a single-point temperature [10]. Reverse transcription-LAMP (RT-LAMP) combines an additional step for detecting the viral RNA. The RT-LAMP tests can be completed quickly, generally in less than an hour, and need minimal equipment, such as a small heat block or water bath for reaction incubation. In addition, the results can be detected by a variety of readouts: fluorescence, turbidity, or color change detection. The colorimetric RT-LAMP assay, in which the results can be simply detected by the naked-eye visualization of color change, offers an alternative and has the potential to be applied in various settings, including low-resource laboratories. The development of RT-LAMP methods can increase the availability of SARS-CoV-2 testing and extend the testing capacity of laboratories. It can be carried out for the simple and rapid testing of SARS-CoV-2, especially when commercial alternatives are not available. This study was designed to develop a colorimetric RT-LAMP using new primers and evaluate its performance for the rapid detection of SARS-CoV-2. The RT-LAMP detection results were compared to those of RT-PCR as a reference using clinical RNA samples. Since SARS-CoV-2 has evolved considerably, new RT-LAMP assays capable of detecting various types of known variants would be useful.

Methodology

Ethics statement

The study protocol was reviewed and approved by the Ethical Committee of Mae Sot Hospital, Ministry of Public Health (MoPH), Thailand (Approval number MSHP *024/2564*), and the study was conducted in accordance with the ethical approval.

RT-LAMP primers

The PrimerExplorer Version 5 software (Eiken Chemical Co., Ltd., Tokyo, Japan) was used to design the RT-LAMP primers targeting the SARS-CoV-2 RNA in the region of *orf8*. The nucleotide sequences and locus of each primer: an outer forward primer (F3), outer backward primer (B3), forward inner primer (FIP), backward inner primer (BIP), loop forward primer (LF), and a loop backward primer (LB) are listed in Table 1.

The viral *orf8* was chosen as a target region for the RT-LAMP since it is highly conserved and specific to SARS-CoV-2. Different SARS-CoV-2 lineages including several Omicron variants were included in sequence analyses. Table 2 presents the *in silico* features of all primers, indicating specificity based on a high sequence homology against SARS-CoV-2 with different lineages and variants, and a low sequence similarity with other microorganisms.

Before preparing a master mix for the RT-LAMP assay, the 10X LAMP primer mixture was prepared in nuclease-free water and stored at -20 °C until use. The final concentrations of each primer in the RT-LAMP reaction were 0.2 µM F3/B3, 1.6 µM FIP/BIP, and 0.4 µM LF/LB.

RT-LAMP reaction

The RT-LAMP reaction was assembled using WarmStart® Colorimetric LAMP 2X Master Mix (New England Biolabs, Ipswich, MA, USA) in a total volume of 25 µL according to the manufacturer's instructions. In each reaction, a volume of 20 µL of the master mix, containing 12.5 µL of WarmStart® Colorimetric LAMP 2X Master Mix, 5 µL of nuclease-free water, and 2.5 µL of 10X primer mix in the concentrations described above, was distributed into 0.2 mL PCR tubes. Each 5 µL of RNA sample was then added immediately just before starting the reaction, followed by mixing, spinning down, and checking the reaction color. The reactions were then incubated at 63 °C for 60 minutes on a thermocycler (Axygen® MaxyGeneII Thermal Cycler, Corning, NY, USA) or heating block and then removed to stop the reaction on ice (60, 63, and 65 °C; 30–90 minutes have been tested in optimization). The

Table 1. RT-LAMP primer sequences targeting *orf8* of SARS-CoV-2 used in this study.

Primers	Nucleotide sequence (5' to 3')	locus*
F3-*ORF8*	TGGTATATTAGAGTAGGAGCTAGA	28026-28049
B3-*ORF8*	AAACAACACGAACGTCATG	28227-28245
FIP *ORF8* (F2+F1c)	TCGATGTACTGAATGGGTGATTTAGTCAGCACCTTTAATTGAATTGTG	28093-28117, 28053-28075
BIP-*ORF8* (B1c+B2)	AATTGCCAGGAACCTAAATTGGGCTCTAAAAAGTCTTCATAGAACGA	28158-28180, 28200-28223
LoopF-*ORF8*	AACCAGCCTCATCCACG	28076-28092
LoopB-*ORF8*	AGTCTTGTAGTGCGTTGT	28182-28199

*Locus reference based on Genbank database, NC_045512.2

end-point results were detected visually for the color of the reaction and simply imaged by a mobile phone camera or office scanner. The color change from pink to yellow indicated a positive result. For validated results, the color of the positive control reaction should be yellow, with the negative control reaction remaining pink.

For positive control, SARS-CoV-2 standard RNA was prepared from SARS-CoV-2 positive samples. The negative control was nuclease-free water without SARS-CoV-2 RNA templates. Positive and negative controls were included in every running assay.

Determination of the analytical sensitivity and specificity

The analytical sensitivity of the developed RT-LAMP assay was assessed using AccuPlex™ SARS-CoV-2 Reference Material Kit (SeraCare, Milford, MA, USA), a reference material of SARS-CoV-2 RNA. This reference material was derived from a construction of Alphavirus recombinant containing SARS-CoV-2 specific sequences of *ORF1a* (open reading frame 1a; spanning nucleotide no. 417–1899 and 3094–3360), *RdRp* (RNA-dependent RNA polymerase; spanning nucleotide no. 13291–13560, 14700–15950 and 18577–19051), *E* (envelope; spanning nucleotide no. 25801–28200), and *N* (nucleocapsid; spanning nucleotide sequence no. 27952–29873) according to SARS-CoV-2 nucleotide sequences in Genbank NC_045512.2. The original available concentration of the reference material was 5,203 copies/mL in viral transport media (VTM).

Table 3. The analytical sensitivity of the RT-LAMP assay for the detection of SARS-CoV-2 RNA.

SARS-CoV-2 RNA concentration (copies/mL)	No. of positive samples/ No. of tested samples
5,000	3/3
4,000	3/3
3,000	3/3
2,500	0/3
1,000	0/3

Table 2. The *in silico* features of RT-LAMP primers based on sequence homology against related microorganisms.

Microorganisms/ Primers	Sequence no.	F3	B3	FIP		BIP		LoopF	LoopB
				F2	F1c	B2	B1c		
Inclusivity						**Homology**			
SARS-CoV-2* Cross-reactivity						> 98			
Human coronavirus 229E	NC_002645.1					< 80.0			
Human coronavirus OC43	NC_006213.1					< 80.0			
Human coronavirus HKU1	NC_006577.2					< 80.0			
Human coronavirus NL63	NC_005831.2					< 80.0			
SARS-coronavirus	NC_004718.3					< 80.0			
MERS-coronavirus	NC_019843.3					< 80.0			
Adenovirus type 1	MH183293.1					< 80.0			
Adenovirus type 2	J01917.1					< 80.0			
Adenovirus type 3	AY599836.1					< 80.0			
Human Metapneumovirus	NC_039199.1					< 80.0			
Parainfluenza virus 1	AF457102.1					< 80.0			
Parainfluenza virus 2	AF533012.1					< 80.0			
Parainfluenza virus 3	NC_001796.2					< 80.0			
Parainfluenza virus 4	NC_021928.1					< 80.0			
Influenza A (H1N1)	FJ966079.1					< 80.0			
Influenza A (H3N2)	KT002533.1					< 80.0			
Influenza B (Victoria)	MN230203.1					< 80.0			
Influenza B (Yamagata)	MK715533.1					< 80.0			
Enterovirus	NC_001472.1					< 80.0			
Respiratory syncytialvirus	NC_001803.1					< 80.0			
Rhinovirus	NC_009996.1					< 80.0			
Chlamydia pneumoniae	NC_005043.1					< 80.0			
Haemophilus influenzae	NZ_LN831035.1			< 80.0				82.35	< 80.0
Legionella pneumophila	NZ_LR134380.1			< 80.0				88.24	< 80.0
Mycobacterium tuberculosis	NC_000962.3					< 80.0			
Streptococcus pneumoniae	NZ_LN831051.1					< 80.0			
Streptococcus pyogenes	NZ_LN831034.1					< 80.0			
Bordetella pertussis	NC_018518.1					< 80.0			
Mycoplasma pneumoniae	NZ_CP010546.1					< 80.0			
Pneumocystis jirovecii	CAKM01000281.1					< 80.0			
Pseudomonas aeruginosa	NC_002516.2					< 80.0			
Staphylococcus epidermis	NZ_CP035288.1					< 80.0			

*Various SARS-CoV-2 lineages/variants were included: Some primers are partially mismatched with the Alpha (G28048T and A28111G; MZ344997.1), Gamma (G28167A; MW642250.1), and Kappa (A28104T; MW966601.1) lineages but all perfectly matched with other lineages including Beta (MW598419.1), Delta (MZ009823.1), Epsilon (MW453103.1), Eta (MW560924.1), Iota (MW643362.1), Lambda (MW850639.1), Zeta (MW523796.1), and various Variants of Concern (VOCs) such as Omicron sublineages: BA.1 (OL672836.1), BA.2 (OM371884.1), BA.2.12.1 (OM958567.1), BA.2.75 (ON990685.1), BA.4 (ON373214.1), BA.5 (ON249995.1), BQ.1.1 (OP412163.1), EG.5.1 (OQ873579.1), and XBB.1.5 (OP790748.1).

The serial dilutions of the above reference RNA material were prepared with the target concentrations listed in Table 3. To determine the analytical limit of detection (LoD), triplicates were performed for each dilution at 63 °C for 60 minutes and tested.

For the analytical specificity evaluation, RNA samples from related coronaviruses and other common respiratory viruses, including Influenza A, H1, and B (Flu A H1, Flu B), Respiratory Syncytial Virus A and B (RSV A, B), and Middle East Respiratory Syndrome (MERS) were tested by the RT-LAMP. The assay was performed as described above, and SARS-CoV-2 RNA was used as the positive control.

Clinical sample processing

RNA sample materials from nasopharyngeal swab (NPS) specimens were used to evaluate the clinical sensitivity, specificity, and accuracy of the RT-LAMP. The specimens were obtained from persons suspected of having COVID-19 as part of standard patient care and stored in a 2 mL VTM. The collected samples were then shipped in sterile containers at 4 °C, delivered to the laboratory, and subjected to rtRT-PCR testing at the diagnostic laboratory of Mae Sot Hospital, MoPH, Thailand. According to the information on circulating SARS-CoV-2 lineages in Thailand, the Delta and the Omicron lineages with different variants were the vast majority in COVID-19 spread at the time of sample collection.

RNA extraction

The total RNA was extracted from 200 µL of the samples using NucleoSpin RNA Virus (Macherey-Nagel GmbH & Co. KG, Germany) as per the manufacturer's instructions. The RNA was eluted in 30 µL of Elution buffer provided with the kit. The resulting RNA was used as RNA templates for detecting SARS-CoV-2 by the RT-LAMP assay.

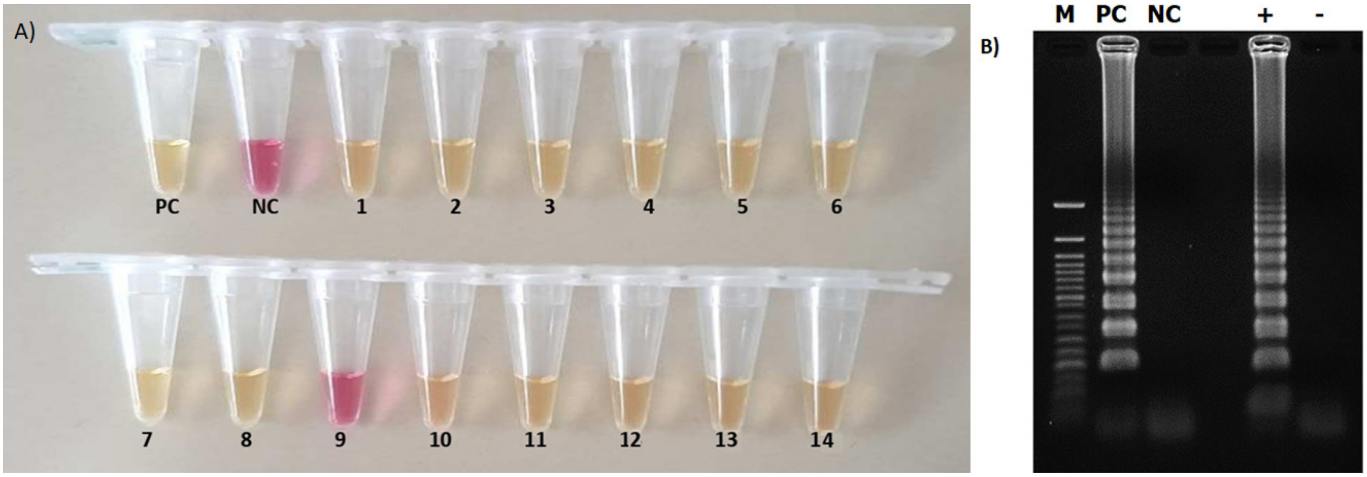
rtRT-PCR reference standard assay

A real-time RT-PCR (rtRT-PCR) assay was used as the reference method for comparative evaluation of the developed RT-LAMP assay. The Molaccu COVID-19 RT-PCR Detection Kit (Zybio Inc., China), approved for in-country COVID-19 diagnosis by the Department of Medical Sciences, MoPH, Thailand, and commercially available as *in vitro* diagnostics (IVD), was used according to the manufacturer's instructions. This assay tests for RNA-dependent RNA polymerase (*RdRp*), nucleocapsid (*N*) genes of SARS-CoV-2, and envelop (*E*) gene of Sarbecovirus. Samples considered positive for SARS-CoV-2 at the amplification cycle threshold (Ct) value ≤ 40 confirm the presence of COVID-19 infection, at least two targets are detected, and the amplification curves are typical S-shape according to the test's instructions. The details of the test kit and the data of LoD (200 antigen units), turnaround time (70 minutes), accuracy (99.53%), clinical sensitivity (99.08%) and clinical specificity (100%) of this test are available on the European Commission website (http://covid-19-diagnostics.jrc.ec.europa.eu/devices/detail/2219).

Clinical evaluation of RT-LAMP

To evaluate the diagnostic performance of the RT-LAMP assay, the RNA extracts were tested by the RT-

Figure 1. Representative results of the colorimetric RT-LAMP assay using a primer set targeting the orf8 of SARS-CoV-2.



A) Results detected by visualization of the color change. PC: positive control; NC: negative Control; 1-14: tested samples. Samples in pink indicate negative and yellow indicate positive results. **B)** Results detected by gel electrophoresis. Lanes M: DNA marker; PC: positive control; NC: negative control. Lane + represents a positive result; Lane – represents a negative result.

LAMP. The testing procedure was carried out as previously described. Briefly, the RT-LAMP reactions were prepared and incubated at 63 °C for 60 minutes in a heating block or conventional thermal cycler. The results of the amplification reactions were visualized for color change with the naked eye. Positive and negative controls were included in all running tests.

Data and statistical analysis

The results were presented in number or percentage formats with 95% confidence intervals (CIs). The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the RT-LAMP assay in relation to the diagnostic test performance were analyzed by comparing the RT-LAMP results to those from the rtRT-PCR test and then calculated.

Results

The new primer set could detect SARS-CoV-2 RNA, with reactions showing a color change from pink to yellow after amplification (Figure 1). The optimal incubation temperature and time for the RT-LAMP reactions were established after testing a range of temperatures at 60, 63, and 65 °C and times from 30–90 minutes. The SARS-CoV-2 viral RNA could be amplified for up to 90 minutes by the RT-LAMP reaction. For the time point > 90 minutes, the color of the negative control sometimes changed from pink to yellow. The optimized conditions for the amplification were finally obtained at 63 °C for 60 minutes with the optimal concentrations of the master mix, primers, RNA samples, and nuclease-free water in tested reactions. Detection of the results was visualized for color change without ambiguity, while gel electrophoresis also clearly revealed a typical banding pattern for the correct amplified RT-LAMP reaction products.

Analytical specificity of the RT-LAMP

The results demonstrated that the primers used in this study were SARS-CoV-2-specific without any cross-reaction against closely related viruses. The color change from pink to yellow could be observed only in the sample containing SARS-CoV-2 within 60 minutes. This result confirmed the virtual analyses of primers based on the *in silico* features for SARS-CoV-2 specificity shown in Table 2.

Analytical sensitivity of the RT-LAMP

The detection limit was evaluated using a set of dilutions for known concentrations of SARS-CoV-2 RNA prepared from AccuPlex™ SARS-CoV-2 Reference Material Kit, a reference material for SARS-CoV-2 RNA. The results from triplicate reactions indicated that the LoD of this RT-LAMP reaction was as low as 3,000 copies/mL shown in 3/3 samples or 15 copies/reaction. Results of the analytical sensitivity of the RT-LAMP assay are summarized in Table 3.

Clinical validation of RT-LAMP detection

A total of 273 RNA samples extracted from NPS clinical specimens with corresponding rtRT-PCR Ct data were received anonymously. Of these, 200 samples were rtRT-PCR positive, and the remaining 73 samples were rtRT-PCR negative for SARS-CoV-2 RNA. The RT-LAMP results could be completely achieved from these tested samples and compared to those of RT-PCR. Visualization of the RT-LAMP results showed that a total of 174/200 (87.00%) rtRT-PCR positive samples were positive by RT-LAMP, and all 73 (100%) rtRT-PCR negative samples were RT-LAMP negative (Table 4). In comparison to rtRT-PCR, the overall diagnostic accuracy, sensitivity, specificity, including PPV and NPV of the RT-LAMP were 90.48% (95% CI: 86.36–93.68%), 87.00% (95% CI: 81.53–91.33%), 100% (95% CI: 95.07–100%), 100% (95% CI: not available), and 73.74% (95% CI: 66.22–80.07%), respectively. A summary of the RT-LAMP results and clinical performance of the RT-LAMP assay in comparison to rtRT-PCR detection is presented in Table 4.

To further evaluate the clinical performance of the RT-LAMP assay, the RT-LAMP results were also compared to those of rtRT-PCR based on the Ct values. The results comparison showed consistency of 100% negative agreement (n = 73), indicating perfect specificity and providing high sensitivity in the majority of positive samples with Ct values ≤ 30 cycles. Based on Ct values ≤ 30 cycles, the sensitivities of the RT-LAMP among those rtRT-PCR positive samples targeting *N*, *RdRp,* and *E* genes were 96.79% (151/156; 95% CI: 92.68–98.95%), 98.03% (149/152; 95% CI: 94.34–99.59%), and 96.25% (154/160; 95% CI: 92.02–98.61%), respectively (Table 4). In samples with Ct values > 30 cycles, the sensitivities of the RT-LAMP declined to 52–50% compared to rtRT-PCR positive results targeting *N* (52.27%, n = 23/44), *RdRp* (52.08%, n = 25/48), and *E* (50.00%, n = 20/40) genes, respectively (Table 4). Compared to those with Ct values ≥ 30–35 cycles, the sensitivities of the RT-LAMP were 56.76% for the *N* gene (n = 21/37), 53.85% for the *RdRp* gene (n = 21/39), and 51.43% (n = 18/35) for the *E* gene, respectively. The declined sensitivities corresponded to the increase in detectable Ct values

across samples. Meanwhile, a few positive samples with Ct values > 35 cycles could be detected by the RT-LAMP, corresponding to the sensitivity reduction to 29% (2/7), 50% (5/10), and 40% (2/5) based on rtRT-PCR detection targeting *N*, *RdRp,* and *E* genes, respectively. The highest sensitivity of the RT-LAMP was 98.03% among samples with Ct values ≤ 30 cycles targeting the *RdRp* gene. Over this high level, the sensitivity of the RT-LAMP dropped off rapidly. In false negative samples, the Ct values (median) with the interquartile range (IQR) were 32.21 (IQR: 30.91-34.08) for the *N* gene, 33.44 (IQR: 31.47-34.2) for the *RdRp* gene, and 32.52 (IQR: 30.25-33.47) for the *E* gene targeting, respectively.

Table 4 shows the varied sensitivity of the RT-LAMP assay based on Ct values. The specificity, accuracy, PPV, and NPV of the RT-LAMP based on rtRT-PCR Ct values are all presented in Table 4.

Discussion

Since the COVID-19 emergence, the rtRT-PCR test has been the primary method to diagnose COVID-19 due to its significant sensitivity, specificity, and speed of detection. To date, the RT-LAMP assay has been approved to be a simple and cost-effective molecular diagnostic for the rapid detection of COVID-19 [4–6]. In this study, a colorimetric RT-LAMP method has been developed and evaluated as an alternative for the rapid detection of SARS-CoV-2 infection in Thailand. The developed RT-LAMP assay demonstrated effective SARS-CoV-2 detection according to its LoD, accuracy, sensitivity, specificity, PPV, and NPV in comparison to rtRT-PCR.

The RT-LAMP assay is fast and easy to perform, requiring few resources and low-cost materials. In this study, the RT-LAMP was developed using a new primer set targeting the highly conserved regions spanning positions 28026-28245 of SARS-CoV-2 *orf8*. The selected regions were free from dominant mutations after checking specific mutations in several variants reported in the SARS-CoV-2 mutation database. Although some primers showed partial mismatches with the genome sequences of Alpha, Gamma, and Kappa lineages, this primer set perfectly matched with those of other lineages which included Delta and different Omicron variants mostly present in Thailand at the time of sample collection. This data suggested that this RT-LAMP may be able to detect many known variants. Recently, we have included about 16,214,741 genomes of SARS-CoV-2 from the Global Initiative on Sharing All Influenza Data (GISAID) database to update sequence analysis. A reduction of the percentage of sequence homology for inclusivity to SARS-CoV-2 suggested that the RT-LAMP results might be impacted by particular mutations in the target regions. In terms of detection speed, our RT-LAMP had a running time up to 60 minutes which was slightly different from 70 minutes by rtRT-PCR using the Molaccu RT-PCR detection kit. Compared to other rtRT-PCR references, the RT-LAMP may have much difference. In addition, its ease of use may contribute to a decrease in hands-on time resulting in a short turnaround time.

This RT-LAMP assay was sensitive and specific for detecting SARS-CoV-2 and comparable to the rtRT-PCR. The evidence of a sensitive RT-LAMP assay for SARS-CoV-2 detection was based on an LoD as low as 15 SARS-CoV-2 RNA copies per reaction, and the diagnostic performance demonstrated its ability to detect SARS-CoV-2 in the majority of positive samples. The overall specificity was 100%, and the 87% sensitivity was within the range of 75–100% compared to those of rtRT-PCR, according to a prior meta-analysis report [11]. Using the WarmStart® Colorimetric LAMP Master mix, a pH-dependent RT-LAMP mixture, a visible color change from pink to yellow could be observed from the release of pyrophosphate and hydrogen ion during amplification,

Table 4. Diagnostic performance of the RT-LAMP assay for the detection of SARS-CoV-2 in clinical specimens in comparison to rtRT-PCR.

rtRT-PCR	Ct (*N*)	RT-LAMP Positive	RT-LAMP Negative	Accuracy (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Positive	≤ 30	151	5	97.82% (94.98-99.29%)	96.79% (92.68-98.95%)	100% (95.07-100%)	100% (NA)	93.59% (86.04-97.19%)
	> 30	23	21					
	Ct (*RdRp*)							
	≤ 30	149	3	98.67% (96.15-99.72%)	98.03% (94.34-99.59%)	100% (95.07-100%)	100% (NA)	96.05% (88.81-98.68%)
	> 30	25	23					
	Ct (*E*)							
	≤ 30	154	6	97.42% (94.48-99.05%)	96.25% (92.02-98.61%)	100% (95.07-100%)	100% (NA)	92.41% (84.73-96.39%)
	> 30	20	20					
	Total	**174**	**26**	**90.48% (86.36-93.68%)**	**87.00% (81.53-91.33%)**	**100% (95.07-100%)**	**100% (NA)**	**73.74% (66.24-80.07%)**
Negative		**0**	**73**					

rtRT-PCR: real-time reverse transcription-quantitative polymerase chain reaction. NA: Not available.

resulting in a decrease of pH in a reaction followed by color change. This pH-sensitive mixture has been applied in a number of RT-LAMP methods described previously for SARS-CoV-2 detection [12–14]. According to the reactions in this study, the pH-sensitive RT-LAMP mixture used had no problem with incompatibility and could show the positive or negative results of the RT-LAMP as described previously [15–17]. It could be simply set up by mixing only a few reaction components at room temperature, enabling its use in limited-resource laboratories.

The sensitivity of different RT-LAMP assays may vary. However, the selected methods for their use should adhere to the appropriate criteria according to the WHO's target product profiles for COVID-19 diagnostics, in which the acceptable sensitivity is at least 80% and specificity at least 97% [18]. They can be considered as an alternative to laboratory-based rtRT-PCR, especially when the rtRT-PCR tests are not available, or confirmation of antigen testing for COVID-19 is required [18]. This RT-LAMP had 87% overall sensitivity compared to rtRT-PCR detection, and therefore, its performance met the criteria priority according to the WHO's recommendation.

The clinical performance of this RT-LAMP method could be further assessed based on rtRT-PCR Ct values. For samples with Ct values ≤ 30 cycles measured by the rtRT-PCR reference, it was found that the RT-LAMP assay missed a positive detection in a few samples, while the highest sensitivity of the RT-LAMP was 98.03% in samples with Ct values ≤ 30 cycles. Meanwhile, the RT-LAMP assay showed a sensitivity reduction of approximately 50% in samples that had tested positive by rtRT-PCR with Ct values > 30 cycles. Overall, a decline in sensitivity was usually observed in samples with high Ct values or low viral load. For further evaluation, it is important to compare the diagnostic performance to those of the commercial or some other RT-LAMP tests. Previously, the clinical sensitivity, specificity, PPV, and NPV of the Loopamp SARS-CoV-2 Detection kit (Eiken Chemical, Tokyo, Japan) were reported to be 78.9, 100%, 100%, and 55.6%, respectively [19]. There have also been other RT-LAMP assays published in the literature with substantially higher sensitivity, shorter testing times, and reduced LoD. For instance, Ali *et al* developed a rapid RT-LAMP with a sensitivity of 96.6% and specificity of 94.7% with a running time of 30 minutes [12]. Thompson and Lei summarized the progress in RT-LAMP development and listed different RT-LAMP methods that enabled COVID-19 detection with a range of test performance [20]. Generally, the clinical sensitivity of each RT-LAMP can be affected by other factors, such as specimens and specimen processing. In addition, the sensitivity may vary according to the reference standard used in the evaluation. These factors should be taken into consideration when comparing tests. Overall, the study's results suggest that this developed RT-LAMP is sensitive and reliable for use in detecting SARS-CoV-2.

The good performance of this RT-LAMP method is presented with some recognized limitations. Since the RNA materials were taken from a routine diagnostic procedure, the potential of the RT-LAMP method for the direct detection of SARS-CoV-2 in raw or unprocessed samples could not be assessed. Furthermore, the capability of the RT-LAMP assay to detect SARS-CoV-2 could not be evaluated in a range of specimens. Lastly, the SAR-CoV RNA material was unavailable for the specificity analysis. Despite these limitations, the assay shows promise and has the potential to expand testing for the rapid and reliable detection of COVID-19 in Thailand.

Conclusions

A sensitive, specific, and accurate colorimetric RT-LAMP method for the rapid detection of SARS-CoV-2 infection was developed in this study. The assay had a LoD of 15 copies of SARS-CoV-2 RNA per reaction, and the greatest clinical sensitivity was 98.03% in samples with Ct values up to 30 cycles. This RT-LAMP can extend the availability of COVID-19 testing with a fast and simple procedure. It can be used particularly when commercial alternatives are not available due to a decline in COVID-19 cases.

Acknowledgements

We gratefully acknowledge Mae Sot Hospital for the collaboration. This study was supported by the Department of Medical Sciences, Ministry of Public Health, Thailand. The funding from the National Research Council of Thailand to JR (no. N35A640044) was acknowledged.

References

1. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R, Niu P, Zhan F, Ma X, Wang D, Xu W, Wu G, Gao GF, Tan W, China Novel Coronavirus Investigating and Research Team (2020) A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med 382: 727-733. doi: 10.1056/NEJMoa2001017.
2. Wang W, Xu Y, Gao R, Lu R, Han K, Wu G, Tan W (2020) Detection of SARS-CoV-2 in different types of clinical specimens. JAMA 323: 1843-1844. doi: 10.1001/jama.2020.3786.

3. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, Bleicker T, Brünink S, Schneider J, Schmidt ML, Mulders DG, Haagmans BL, van der Veer B, van den Brink S, Wijsman L, Goderski G, Romette JL, Ellis J, Zambon M, Peiris M, Goossens H, Reusken C, Koopmans MP, Drosten C (2020) Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill 25: 2000045. doi: 10.2807/1560-7917.ES.2020.25.3.2000045.
4. Fernandes RS, de Oliveira Silva J, Gomes KB, Azevedo RB, Townsend DM, de Paula Sabino A, Branco de Barros AL (2022) Recent advances in point of care testing for COVID-19 detection. Biomed Pharmacother 153: 113538. doi: 10.1016/j.biopha.2022.113538.
5. Song Q, Sun X, Dai Z, Gao Y, Gong X, Zhou B, Wu J, Wen W (2021) Point-of-care testing detection methods for COVID-19. Lab Chip 21: 1634-1660. doi: 10.1039/d0lc01156h.
6. Yüce M, Filiztekin E, Özkaya KG (2021) COVID-19 diagnosis -a review of current methods. Biosens Bioelectron 172: 112752. doi: 10.1016/j.bios.2020.112752.
7. Cubas-Atienzar AI, Kontogianni K, Edwards T, Wooding D, Buist K, Thompson CR, Williams CT, Patterson EI, Hughes GL, Baldwin L, Escadafal C, Sacks JA, Adams ER (2021) Limit of detection in different matrices of 19 commercially available rapid antigen tests for the detection of SARS-CoV-2. Sci Rep 11: 18313. doi: 10.1038/s41598-021-97489-9.
8. Khandker SS, Nik Hashim NHH, Deris ZZ, Shueb RH, Islam MA (2021) Diagnostic accuracy of rapid antigen test kits for detecting SARS-CoV-2: A systematic review and meta-analysis of 17,171 suspected COVID-19 patients. J Clin Med 10: 3493. doi: 10.3390/jcm10163493.
9. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28: E63. doi: 10.1093/nar/28.12.e63.
10. Nagamine K, Hase T, Notomi T (2002) Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes 16: 223-229. doi: 10.1006/mcpr.2002.0415.
11. Subali AD, Wiyono L (2021) Reverse transcriptase loop mediated isothermal amplification (RT-LAMP) for COVID-19 diagnosis: a systematic review and meta-analysis. Pathog Glob Health 115: 281-291. doi: 10.1080/20477724.2021.
12. Ali H, Alkhaursi K, Holton T (2022) Development of a colorimetric RT-LAMP assay for the detection of SARS-COV-2 isolated from Oman. J Infect Dev Ctries 16: 952-958. doi: 10.3855/jidc.15377.
13. Rivas-Macho A, Sorarrain A, Marimón JM, Goñi-de-Cerio F, Olabarria G (2023) Extraction-free colorimetric RT-LAMP detection of SARS-CoV-2 in saliva. Diagnostics (Basel) 13: 2344. doi: 10.3390/diagnostics13142344.
14. Raddatz BW, Rabello FJ, Benedetti R, Steil GJ, Imamura LM, Kim EYS, Santiago EB, Hartmann LF, Predebon JV, Delfino BM, Nogueira MB, Dos Santos JS, da Silva BG, Nicollete DRP, Almeida BMM, Rogal SR Jr, Figueredo MVM (2023) Clinical validation of a colorimetric loop-mediated isothermal amplification using a portable device for the rapid detection of SARS-CoV-2. Diagnostics (Basel) 13: 1355. doi: 10.3390/diagnostics13071355.
15. Tanner NA, Zhang Y, Evans TC Jr (2015) Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes. Biotechniques 58: 59-68. doi: 10.2144/000114253.
16. Poole CB, Li Z, Alhassan A, Guelig D, Diesburg S, Tanner NA, Zhang Y, Evans TC Jr, LaBarre P, Wanji S, Burton RA, Carlow CK (2017) Colorimetric tests for diagnosis of filarial infection and vector surveillance using non-instrumented nucleic acid loop-mediated isothermal amplification (NINA-LAMP). PLoS One 12: e0169011. doi: 10.1371/journal.pone.0169011.
17. Huang WE, Lim B, Hsu CC, Xiong D, Wu W, Yu Y, Jia H, Wang Y, Zeng Y, Ji M, Chang H, Zhang X, Wang H, Cui Z (2020) RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2. Microb Biotechnol 13: 950-961. doi: 10.1111/1751-7915.13586.
18. Dinnes J, Deeks JJ, Adriano A, Berhane S, Davenport C, Dittrich S, Emperador D, Takwoingi Y, Cunningham J, Beese S, Dretzke J, Ferrante di Ruffano L, Harris IM, Price MJ, Taylor-Phillips S, Hooft L, Leeflang MM, Spijker R, Van den Bruel A; Cochrane COVID-19 Diagnostic Test Accuracy Group (2020) Rapid, point-of-care antigen and molecular-based tests for diagnosis of SARS-CoV-2 infection. Cochrane Database Syst Rev 8: CD013705. doi: 10.1002/14651858.CD013705.
19. Higashimoto Y, Ihira M, Kawamura Y, Inaba M, Shirato K, Suzuki T, Hasegawa H, Kageyama T, Doi Y, Hata T, Yoshikawa T (2023) Dry loop-mediated isothermal amplification assay for detection of SARS-CoV-2 from clinical specimens. Fujita Med J 9: 84-89. doi: 10.20407/fmj.2022-003.
20. Thompson D, Lei Y (2020) Mini review: recent progress in RT-LAMP enabled COVID-19 detection. Sens Actuators Rep 2: 100017. doi: 10.1016/j.snr.2020.100017.

Corresponding author

Benjawan Phetsuksiri, PhD
88/7 Department of Medical Sciences,
Ministry of Public Health, Nonthaburi, Thailand.
Tel: +66 025801567
Fax: +66 029659700
Email: benjapsk@health.moph.go.th

Conflict of interests: No conflict of interests is declared.