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Performance of colorimetric reverse transcription loop-mediated isothermal amplification as a diagnostic tool for SARS-CoV-2 infection during the fourth wave of COVID-19 in Thailand

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ABSTRACT

Background: COVID-19, which is caused by SARS-CoV-2 and its variants, poses an ongoing global threat, particularly in low-immunization coverage regions. Thus, rapid, accurate, and easy-to-perform diagnostic methods are in urgent demand to halt the spread of the virus.

Objectives: We aimed to validate the clinical performance of the FastProof 30 min-TTR SARS-CoV-2 reverse transcription loop-mediated isothermal amplification (RT-LAMP) method using leftover RNA samples extracted from 315 nasopharyngeal swabs. The sensitivity and specificity of RT-LAMP were determined in comparison with reverse transcriptase–polymerase chain reaction (RT-PCR).

Results: Of 315 nasopharyngeal swabs, viral RNA was detected in 154 samples (48.9%) by RT-PCR assay. Compared with RT-PCR, overall sensitivity and specificity of RT-LAMP were 81.82% (95% CI: 74.81–87.57) and 100% (95% CI: 97.73–100), respectively. A 100% positivity rate was achieved in samples with cycle threshold (Ct) <31 for RT-PCR targeting the *ORF1ab* gene. However, samples with Ct >31 accounted for false-negative results by RT-LAMP in 28 samples.

Conclusions: RT-LAMP reliably detected viral RNA with high sensitivity and specificity and has potential application for mass screening of patients with acute COVID-19 infection when viral load is high.

Introduction

COVID-19 is an acute infection of the respiratory tract caused by a recently emerged coronavirus, SARS-CoV-2, which was first detected in Wuhan, China, in late 2019, and continues to cause major public health concerns. The virus spread worldwide and has been declared a pandemic because there were more than 194 million confirmed cases and more than 4 million deaths in 222 countries as of July 2021 (World Health Organization, 2021). Currently, there are different platforms of COVID-19 vaccines available which have been distributed globally. Yet, the number of new confirmed cases continues to increase in many countries, especially in regions with low-vaccination rates.

In Thailand, the number of daily positive COVID-19 cases and deaths remain high because of insufficient vaccine coverage and the impact of SARS-CoV-2 variants. At the time of manuscript preparation, the fourth COVID-19 wave driven by the delta variant was surging. This variant is highly contagious and rapidly transmitted among vaccinated and unvaccinated people. However, accurate and reliable diagnostic tests for SARS-CoV-2 infection and nonpharmaceutical measures could decelerate the spread of SARS-CoV-2 by allowing faster identification and separation of infected individuals from uninfected individuals. Reverse transcriptase–polymerase chain reaction (RT-PCR), a gold-standard laboratory test, has been used routinely for COVID-19 diagnosis since the beginning of the outbreak because of its high sensitivity and specificity (Corman et al, 2020). However, the method is time consuming and requires costly equipment and trained personnel, which is not applicable for mass testing during the fourth-wave COVID-19 crisis (Feng et al, 2020; Shen et al, 2020).

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Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is a rapid, reliable alternative molecular test. This method amplifies specific genes at a constant temperature of 60°C to 65°C for a duration of 30 minutes. RT-LAMP reactions can be detected through various readouts, including gel electrophoresis, turbidity, fluorescence, and visual color changes (Amaral et al, 2021; Ganguli et al, 2020; Inaba et al, 2020; Nawattanapaiboon et al, 2021; and Silva et al, 2021). Therefore, RT-LAMP could overcome the drawbacks of RT-PCR in terms of fast turnaround time and affordable reagents and instruments, which would enable large-scale rapid testing (Jiang et al, 2020). Colorimetric RT-LAMP has been established for naked eye observation. This approach has been well validated in real-life clinical settings and was demonstrated to achieve 95% to 100% specificity and 90% to 100% sensitivity, based on the cycle threshold (Ct) values representing viral RNA levels (Kitagawa et al, 2020; Nawattanapaiboon et al, 2021). Decreased sensitivity was frequently found in samples with low viral RNA copies (Silva et al, 2021; Subsoontorn et al, 2020). Colorimetric FastProof RT-LAMP was developed as an alternative rapid, reliable, and cost-effective diagnostic test for COVID-19, which provides support for mass testing and active case detection in regions with low resources. In this study, we therefore validated the performance of the colorimetric RT-LAMP method compared with the gold-standard RT-PCR method using the same 315 COVID-19–suspected clinical samples.

Materials and Methods

Clinical specimen

Nasopharyngeal swabs were obtained from 315 patients with suspected COVID-19 and preoperative patients at Vajira Hospital, Navamindradhiraj University, Bangkok, Thailand, in July 2021. Briefly, nasopharyngeal swabs collected from acute respiratory infection clinic were subjected to RNA extraction and followed by RT-PCR in the laboratory. After completing the routine operation, all RT-PCR positive RNA samples regardless of Ct values and 5% of RT-PCR negative RNA samples were taken daily for RT-LAMP testing. The study protocol was approved by the institutional review board of the Faculty of Medicine Vajira Hospital, Navamindradhiraj University (study code 126/64 E); Certificate of approval (COA): 131/2564.

Sample collection

The specimens were collected in 2 mL viral transport media (VTM) (Dewei Medical Equipment Co, Ltd, China) and were immediately transported at a temperature of 2°C to 8°C to the Biomolecular Laboratory at Vajira Hospital.

RNA extraction

RNA was extracted from 200 µL of VTM samples using the Zybio Nucleic Acid Extraction Kit (magnetic bead method) (Zybio Inc, China) according to the manufacturer's instructions. From each sample, 50 µL of viral RNA was eluted and used as a template for detecting SARS-CoV-2 infection by RT-PCR and RT-LAMP.

Reverse transcriptase–polymerase chain reaction

RT-PCR detection of the *ORF1ab* and *N* genes of SARS-CoV-2 was performed using Sansure Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing) (Sansure Biotech, China) according to the manufacturer's instructions. Briefly, the final reaction volume of 40 µL consisted of 26 µL 2019-nCoV-PCR Mix, 4 µL 2019-nCoV-PCR-Enzyme Mix, and 10 µL extracted RNA. The Slan 96P Real Time PCR System (Sansure Biotech, China) was used for amplification. The amplification reaction consisted of one cycle at 50°C for 30 minutes and one cycle at 95°C for 1 minute for reverse transcription and reverse transcription denaturation, respectively. Next, 45 cycles were repeated at 95°C for 15 seconds and 60°C for 30 seconds with DNA polymerase. The result was analyzed using ABI 7500 software, in which a Ct value <40 for both target genes was defined as a positive result.

Reverse transcription loop-mediated isothermal amplification

RT-LAMP detection of the *N* genes of SARS-CoV-2 and human RNase *P* gene as an internal control was performed using the FastProof 30 min-TTR SARS-CoV-2 RT-LAMP Kit (Zenostic Co, Ltd., Thailand) according to the manufacturer's instructions. The total volume was 20 µL, which consisted of 10 µL Color LAMP-TTR, 5 µL primer mix-TTP, and 5 µL extracted RNA sample. The reaction was incubated at 65°C for 30 minutes, in a T100 Thermal Cycler (Bio-Rad Laboratories, Inc, Hercules, California). A positive result was determined by observing the colors of the reaction changes from pink to yellow. Positive and negative controls were used in all assays.

Statistics

Descriptive data were presented in the following formats: percentage, median, range, mean, and SD. The sensitivity and specificity of RT-LAMP in detecting SARS-CoV-2 were analyzed using GraphPad Prism 6.0 (GraphPad Prism Software Inc, San Diego, California) and MedCalc's diagnostic test evaluation, an online statistical calculator.

Results

Nasopharyngeal swabs were collected from a total of 315 COVID-19 suspected cases and preoperative patients in July 2021 at Vajira Hospital, a tertiary hospital in Bangkok, Thailand. Patient characteristics were retrospectively reviewed from medical records as shown in Table 1. The median age of the study population was 40 (range: <1–92) years; 186 were women (59%) and 129 were men (41%). Of 315 subjects, 196 presented illnesses, 110 had no symptoms, and 9 were underreported. Common symptoms included fever, cough, sore throat, runny nose, muscle aches, headache, loss of taste or smell, diarrhea, difficulty breathing, and pneumonia, which appeared 0 to 30 days after exposure or direct contact with COVID-19 confirmed cases. The diagnosis of COVID-19 was confirmed by the gold-standard RT-PCR technique. According to RT-PCR results, 154 samples (48.9%) were positive and 161 were negative (51.1%). The average Ct values in RT-PCR-positive cases ranged from 14.74 to 39.96 for the *ORF1ab* gene and 10.95 to 38.14 for the *N* gene. Samples with Ct values >40 for both *ORF1ab* and *N* genes were reported as negative for COVID-19. The average Ct value of the *ORF1ab* gene was 3.4 cycle higher than that of the *N* gene (Table 1). This result indicated that the *ORF1ab* gene was less sensitive than the *N* gene for detecting SARS-CoV-2. Among 154 RT-PCR positive cases, 124 cases (80.5%) were symptomatic, and 30 cases (19.5%) were asymptomatic (Table S1).

To validate the performance of the colorimetric RT-LAMP method, all 315 leftover RNA samples were assessed by RT-LAMP, of which, 126 and 189 samples were found to be positive and negative, respectively. Compared with RT-PCR, the overall sensitivity and specificity of RT-LAMP were 81.82% (95% CI: 74.81–87.57) and 100% (95% CI: 97.73–100), respectively (Table 2). Of 154 positive samples by RT-PCR, 126 were detected by colorimetric RT-LAMP. Concordant positive samples had a median Ct of 23.87 (range:

Table 1
Characteristics of patients in this study

Characteristics	Results
Samples, n	315
Age, median (range)	40 y (<1–92 y)
Sex, n (%)	
Male	129 (41.0)
Female	186 (59.0)
Clinical presentation, n (%)	
Symptomatic	196 (62.2)
Asymptomatic	110 (34.9)
No information	9 (2.9)
Mean of duration of onset to test (range), d	4.3 (0–30)
Results of RT-PCR assay, n (%)	
Negative	161 (51.1)
Positive	154 (48.9)
Mean ±SD of Ct value of *ORF1ab* gene (minimum–maximum)	26.39 ± 6.77 (14.74–39.96)
Mean ± SD of Ct value of *N* gene (minimum–maximum)	22.98 ± 6.64 (10.95–38.14)
Results of RT-LAMP (FastProof 30 min-TTR SARS-CoV-2 RT-LAMP Kit), n (%)	
Positive	126 (40)
Negative	189 (60)

Table 2
Sensitivity and specificity of reverse transcription loop-mediated isothermal amplification (FastProof 30 min-TTR SARS-CoV-2 RT-LAMP Kit)

			RT-LAMP				
		Ct value	Positive(n)	Negative (n)	Total (n)	Sensitivity/specificity (%)	95% CI (%)
RT-PCR	Positive	<25	76	0	76	100.00	95.26–100.00
		25–31	33	0	33	100.00	89.42–100.00
		31–36	14	12	26	53.85	33.37–73.41
		36–40	3	16	19	15.79	3.38–39.58
		Total	126	28	154	81.82	74.8–87.57
	Negative	negative	0	161	161	100.00	97.73–100.00

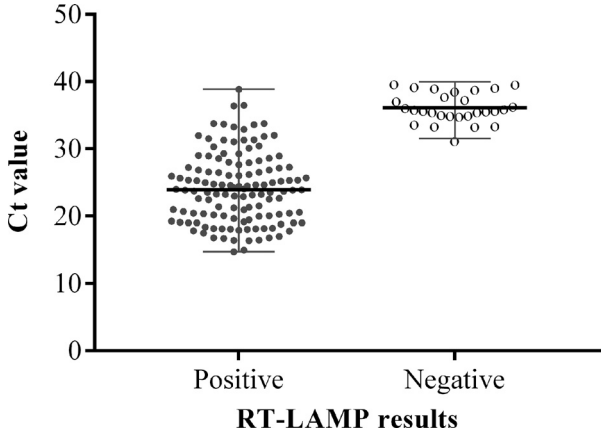

Figure 1. Reverse transcription loop-mediated isothermal amplification results according to the cycle threshold value of viral RNA.

14.74–38.87), whereas the 28 samples that were negative in RT-LAMP had a median Ct of 36.09 (rang: 31.52–39.96) (Fig. 1).

The performance of RT-LAMP in detecting SARS-CoV-2 in symptomatic patients versus asymptomatic patients was further evaluated. Among the 126 true positive results, RT-LAMP was able to detect SARS-CoV-2 in symptomatic patients with 87.10% sensitivity, whereas the sensitivity was slightly decreased in asymptomatic patients (60%). For 28 false negative results, RT-LAMP could not detect SARS-CoV-2 in 16 and 12 symptomatic and asymptomatic patients, respectively (Table 3). These results suggested that RT-LAMP assay exhibited high accuracy for determining SARS-CoV-2 infection, especially in symptomatic patients.

The correlation between the positivity rate by RT-LAMP and 4 Ct ranges was explored (Table 2). All samples with Ct values <31 in RT-PCR had a 100% positivity rate by RT-LAMP. A decrease in the positivity rate of Ct values ≥31, which declined to 53.85%, was obvious; whereas for samples with Ct values ≥36, the positivity rate decreased to 15.79%. The 28 samples that were discordant with the RT-PCR results were false negatives due to Ct values >31 (range: 31.52–39.96). Our finding demonstrated that RT-LAMP could effectively detect the SARS-CoV-2 RNA in samples with low to moderate Ct values.

Discussion

Rapid identification of infection is the key to control the spread of COVID-19. Thus, fast, simple, and reliable approaches are needed to speed up disease screening and surveillance in large-scale testing. RT-LAMP is a promising method for achieving rapid, accurate, and easy-to-perform diagnostic methods. However, the quality of RT-LAMP depends on the quality of reagents, primer design, and target genes of the SARS-CoV-2 that are used for detection. Importantly, the RT-LAMP test kit for the SARS-CoV-2 diagnosis should be authorized by the US Food and Drug Administration (FDA) or the regulatory agencies in the country of production before dispersing through the market. Low quality of RT-LAMP test kit could result in the high rate of false-positive and false-negative diagnoses as evidenced by Freire-Paspuel and Garcia-Bereguiain study (Freire-Paspuel and Garcia-Bereguiain, 2021). Therefore, the clinical performance of the RT-LAMP test kit needs to be investigated thoroughly. FastProof 30 min-TTR SARS-CoV-2 RT-LAMP Kit was developed by a Thai startup company and was recently approved by the Thai FDA. It is a colorimetric RT-LAMP assay kit that has minimal cost and simply reads the results by observing changes in color from pink to yellow within 30 minutes of the reaction. Launching this test kit in the in vitro diagnostics market could eliminate concerns about delayed international reagent shipping and the costs of expensive RT-PCR tests, which supports the feasibility of using colorimetric

Table 3
Sensitivity of reverse transcription loop-mediated isothermal amplification (FastProof 30 min-TTR SARS-CoV-2 RT-LAMP Kit) in symptomatic and asymptomatic subjects

Condition	RT-LAMP results				
	Positive (n)	Negative (n)	Total (n)	Sensitivity (%)	95% CI (%)
Symptomatic subjects	108	16	124	87.10	79.89–92.44
Asymptomatic subjects	18	12	30	60.00	40.60–77.34
Total	126	28	154	81.82	74.8–87.57

RT-LAMP in low-to-middle income countries where immunization coverage may be poor.

In this study, we validated the performance of this colorimetric RT-LAMP kit compared with RT-PCR to assure that it could be used as an alternative diagnostic test and/or screening test for COVID-19 detection in the real-world clinical setting. The overall sensitivity and specificity of RT-LAMP were 81.82% and 100%, respectively. The greatest sensitivity of 100% was shown at Ct values <31, which dramatically decreased to 15.79% at Ct values >36, indicating that for the low viral load, RT-LAMP was much less sensitive. The correlation between the Ct values of patients and the disease severity revealed that a Ct values >30 was associated with low mortality and Ct value >33 may not potentially transmit diseases (Magleby et al, 2021; La Scola et al, 2020). In contrast, low viral loads probably appeared in the preincubation period and later increased within a few days of testing negative.

In our study, the RT-LAMP assay was three times faster and cheaper than the gold-standard RT-PCR, which helped to minimize time and cost in diagnosis. However, this kit requires staff without vision problems and laboratory equipment for the RNA extraction step and preparation of the reaction mixture; thus, it may not be accessible to remote health clinics. The SARS-CoV-2 Rapid Antigen Test (RAT) kit, which is based on immunochromatography, became popular in many countries, including Thailand. The RAT kit is a fast and simple test, which is more accessible for patients than the RT-LAMP. Currently, there are large numbers of SARS-CoV-2 RAT kits with varying quality available in the market. Even the kits produced by well-known manufacturers had lower efficacy for samples with Ct values of RT-PCR >24 to 28 (Platten et al, 2021). The performance of Standard Q COVID-19 Ag kit was evaluated in a Thai health care facility showing 98.33% sensitivity and 98.73% specificity. This high sensitivity could be due to adding the extra step of reducing viscosity in samples. In addition, two-thirds of the tested samples had Ct values <28 (Chaimayo et al, 2020). Thus, the RAT has both advantages and drawbacks: it is easy to perform, and affordable at-home test kits are preferable, but the drawbacks are the high rate of false-negative results arising from low viral loads and the lack of proper specimen processing. Still, RT-LAMP has advantages over RAT in terms of sensitivity and specificity. The RT-LAMP kit's sensitivity and specificity in our setting are better than RAT because the overall sensitivity and specificity of RAT in previous reports were 68.4% and 99.4%, respectively (Khandker et al, 2021).

Our findings are concordant with several colorimetric RT-LAMP studies that reported sensitivity of 70% to 90% and specificity of 99% to 100% (Nawattanapaiboon et al, 2021; Silva et al, 2021; Subsoontorn et al, 2020). Similarly, at higher Ct values (representing low viral RNA levels), lower RT-LAMP sensitivity was observed. Variations of sensitivity in different studies could be explained by the number of positive samples with high Ct values ranging from 30 to 40 included in each study. Dao Thi *et al.* reported that approximately a third of positive samples had Ct values of 30 to 40, which accounted for a decreased sensitivity to 70% (Dao Thi et al., 2020). In contrast, Nawattanapaiboon *et al.* reported 95.74% sensitivity because most of the positive samples (42/47) had Ct values <30 (Nawattanapaiboon et al., 2021). In our study, the number of positive samples with Ct values of 30 to 40 is comparable with the study of Dao Thi et al; 45 of 154 positive samples had Ct values >31. Thus, the number of positive cases among the tested samples could be the best explanation for our 81.82% RT-LAMP sensitivity. Besides the number of tested samples, the sensitivity of colorimetric RT-LAMP assays depends on several factors, such as the limit of detection, type of specimen, sample quality, level of viral RNA in the sample and time of specimen collection, and how it is processed. The presence of the patient matrix in clinical samples was responsible for reducing the detection rate of the RT-LAMP assay (Silva et al, 2021); the unskilled nasopharyngeal swab collections resulted in viral loads that were too low despite the samples being collected during the early phase of infection (Mallett et al, 2020; Zou et al, 2020); and the pH >8 of the elution buffer in the RNA purification step interfered with the color changes in the RT-LAMP reactions. These issues could contribute to misinterpretation (Huang et al, 2020; Nawattanapaiboon et al, 2021). A lack of actual viral loads in RNA copies per microliter unit is a limitation of this study. Direct swab-to-RT-LAMP without an RNA purification step as well as testing with other respiratory specimens such as saliva or sputum should be performed in further studies. Considered together, colorimetric RT-LAMP exhibited satisfactory performance. The assay has good sensitivity and specificity and is able to detect high-to-moderate viral RNA levels, which frequently appear at the early phase of symptom onset.

Conclusion

This study demonstrated that the FastProof 30 min-TTR SARS-CoV-2 RT-LAMP Kit provides rapid and simple molecular testing that has high specificity and sensitivity. It has the potential to be used as an alternative screening tool to help speed up SARS-CoV-2 diagnosis. However, because the sensitivity of RT-LAMP at low viral loads is not comparable with that of RT-PCR, negative results could not rule out SARS-CoV-2 infection; hence, negative results in patients with COVID-19 symptoms should be confirmed by RT-PCR.

Conflict of interest

The authors declare no conflict of interest.

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Ethical approval

The study protocol was approved by the institutional review board of the Faculty of Medicine Vajira Hospital, Navamindradhiraj University (study code 126/64 E); COA: 131/2564.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2021.12.351.

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