Brief Report

A Single-Tube Colorimetric Loop-Mediated Isothermal Amplification for Rapid Detection of SARS-CoV-2 RNA

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Abstract: Since SARS-CoV-2 is a highly transmissible virus, a rapid and accurate diagnostic method is necessary to prevent virus spread. We aimed to develop and evaluate a new rapid colorimetric reverse transcription loop--mediated isothermal amplification (RT-LAMP) assay for SARS-CoV-2 detection in a single closed tube. Nasopharyngeal and throat swabs collected from at-risk individuals testing for SARS-CoV-2 were used to assess the sensitivity and specificity of a new RT-LAMP assay against a commercial qRT-PCR assay. Total RNA extracts were submitted to the RT-LAMP reaction under optimal conditions and amplified at 65 °C for 30 min using three sets of specific primers targeting the nucleocapsid gene. The reaction was detected using two different indicator dyes, hydroxynaphthol blue (HNB) and cresol red. A total of 82 samples were used for detection with HNB and 94 samples with cresol red, and results were compared with the qRT-PCR assay. The sensitivity of the RT-LAMP-based HNB assay was 92.1% and the specificity was 93.2%. The sensitivity of the RT-LAMP-based cresol red assay was 80.3%, and the specificity was 97%. This colorimetric feature makes this assay highly accessible, low-cost, and user-friendly, which can be deployed for massive scale-up and rapid diagnosis of SARS-CoV-2 infection, particularly in low-resource settings.

Keywords: COVID-19; SARS-CoV-2 *N* gene; RT-LAMP; hydroxynaphthol blue; cresol red

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1. Introduction

The outbreak of a novel severe acute respiratory syndrome coronavirus (SARS-CoV-2) in late 2019 has led to a global coronavirus disease 2019 (COVID-19) pandemic. As of 8 May 2023, the number of global coronavirus cases had exceeded 765 million, resulting in 6.9 million fatalities across nearly 200 countries [1]. The COVID-19 pandemic has emphasized the need for rapid and accurate diagnostic methods for the detection of SARS-CoV-2 to limit the spread of the virus. Presently, several rapid antigen test kits (ATKs) can be used for massive detection and are cheap compared to molecular tests. However, ATK has a lower sensitivity than molecular tests [2]. Real-time reverse transcription polymerase

chain reaction (RT-PCR) has been widely adopted as the gold standard for the molecular diagnosis of SARS-CoV-2 due to its high specificity and sensitivity. However, most real-time RT-PCR assays provide results in over 3 h. Furthermore, they require expensive equipment and specialized laboratory facilities, which hinder their implementation, particularly in remote and resource-limited settings.

One recent technique, reverse transcription loop-mediated isothermal amplification (RT-LAMP), has emerged as a promising technique for the rapid detection of various pathogens, including influenza [3], Middle East respiratory syndrome coronavirus [4], hepatitis C virus [5], and Ebola [6]. Unlike PCR, LAMP operates under isothermal conditions and can be performed using a regular water bath or heat block that maintains a constant temperature. The LAMP reaction can be completed within a short time frame (15–60 min) and offers high specificity [7]. Several RT-LAMP-based assays have also been designed for SARS-CoV-2 detection, targeting different regions of the viral genome with distinct primer sets [8–13]. Some studies have employed turbidity or fluorescence detection, which still requires specialized and costly equipment not available in resource-limited settings [9,11,12]. RT-LAMP assays for SARS-CoV-2 RNA detection have been developed using pH indicator dyes [14] or hydroxynaphthol blue (HNB) [15] to remove the need for expensive equipment or instruments. Also, colorimetric LAMP kits are currently commercialized; however, these kits are relatively expensive [8,10,12,16].

We present herein the development of a rapid colorimetric RT-LAMP assay for the detection of SARS-CoV-2 in a single closed tube and the evaluation of its sensitivity and specificity on clinical samples. A key advantage of our assay is that the reaction color change can be easily observed with the naked eye, eliminating the need for specialized equipment or complex detection methods.

2. Materials and Methods

2.1. Sample Collection, Processing and Storage

Nasopharyngeal and throat swabs were collected from 176 symptomatic individuals who underwent SARS-CoV-2 testing at the Faculty of Associated Medical Sciences-Clinical Service Center (AMS-CSC), Chiang Mai University (CMU), including 82 individuals coming between May 2020 and December 2021 and 94 between June 2021 and March 2022. All samples used in this study were residual samples. These samples were stored at −70 °C until testing. This study was approved by the Faculty of Associated Medical Sciences Ethic Committee (AMSEC-64EM-028) and authorized by the Institutional Biosafety Committee of the Research Institute for Health Sciences, CMU (CMUIBC0363002).

2.2. RNA Extraction

Total RNA was extracted from 200 µL of swab samples using either the QIAamp Viral RNA Mini Kit (QIAGEN, Germany) or the Nucleic Acid Extraction Kit (Zybio, Chongqing, China), following the manufacturer's instructions. From the resulting 60 µL total RNA extract, 5 µL were subjected to the qRT-PCR SARS-CoV-2 assay (DaAn GENE Co., Ltd., Guangzhou, China) on the automated abCyclerQ instrument (ATI Biotech, Singapore), where the cycle threshold (CT) value above 40 is considered undetectable. Another 5 µL of viral RNA was tested for SARS-CoV-2 RNA with the RT-LAMP assay.

2.3. RT-LAMP Assay

The RT-LAMP primers targeting the SARS-CoV-2 nucleocapsid gene (*N* gene) were derived from the previous study [17]. The two outer primers (forward outer primer "NC1 F3" and backward outer primer "NC1 B3"), two inner primers (forward inner primer "NC1 FIP" and backward inner primer "NC1 BIP"), and two loop primers (loop forward primer "NC1 LF" and loop backward primer "NC1 LB") were used in the RT-LAMP reaction. The oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

2.3.1. RT-LAMP Assay Based on HNB

Colorimetric detection with a metal indicator dye, hydroxynaphthol blue (HNB): a 25 µL reaction was prepared as follows: 1× isothermal amplification buffer (New England Biolabs, MA, USA), 8 mM $MgSO_4$, 1.4 mM deoxynucleotide (dNTP) mix, 1.6 mM of FIP and BIP primers, 0.2 mM F3 and B3 primers, 0.4 mM LF and LB primers, 7.5 U WarmStart RTx Reverse Transcriptase (New England Biolabs, MA, USA), 8 U *Bst* 2.0 WarmStart DNA Polymerase (New England Biolabs, MA, USA), 120 µM hydroxynaphthol blue-HNB (Honeywell, Charlotte, NC, USA), and 5 µL RNA template. The amplification reaction was carried out at 65 °C for 30 min and then terminated at 80 °C for 10 min. Positive results were indicated by a color change to sky blue, while negative results retained the purple color. To confirm the results, 5 µL of RT-LAMP products were electrophoresed on a 2% *w/v* agarose gel, stained with RedSafe (iNtRON Biotechnology, Gyeonggi-do, Republic of Korea), and bands were visualized with a UV transilluminator. A positive RT-LAMP product displayed a characteristic ladder-like pattern with multiple bands of varying sizes. HNB is a metal ion indicator commonly used in the RT-LAMP reaction. It undergoes a color change from purple to sky blue in the presence of magnesium pyrophosphate, a byproduct of the RT-LAMP reaction [18]. A potential limitation of HNB is that the color change from purple to sky blue may be challenging to discriminate with the naked eye, particularly in certain lighting conditions or for individuals with color vision deficiencies. To address this issue, we used cresol red dye as an alternative indicator in the RT-LAMP assay.

2.3.2. RT-LAMP Assay Based on Cresol Red

Colorimetric detection with a pH indicator dye, cresol red: A 25 µL reaction was prepared as follows: 10 mM $(NH_4)2SO_4$, 50 mM KCl, 0.1% *v/v* Tween-20, 8 mM $MgSO_4$, 1.4 mM dNTP mix. The pH of the mixture was adjusted to 8–9 using 1 M KOH, as determined by pH paper. Primers and enzymes were added to the reaction at the same concentrations as the HNB-based assay. In this reaction, 100 µM cresol red was included instead of HNB as the pH indicator dye. The amplification reaction was performed under the same conditions as the HNB-based assay. Cresol red undergoes a color transition from pink to yellow due to a decrease in pH level following the release of protons during amplification [19]. A color change to yellow indicated a positive result, while a pink color indicated a negative result.

2.4. Statistical Analysis

The specificity and sensitivity were calculated using variables with binary values between the RT-LAMP assays based either on HNB or cresol red and the qRT-PCR assay. GraphPad was used to calculate the 95% confidence intervals (95% CI) for the proportion.

3. Results

3.1. Optimization of RT-LAMP Assay Based on HNB

The RT-LAMP assay based on HNB dye was optimized by investigating different parameters, including amplification time and $MgSO_4$ and HNB dye concentrations. Figure 1 depicts the results of these optimization experiments. To determine the optimal amplification time, the RT-LAMP assay was conducted at 10-min intervals, from 20 to 60 min at 65 °C. In the positive control (Figure 1A), a color change from purple to sky blue was observed from 20 min onward. However, non-specific amplifications resulting in a color change were observed in the no template control (NTC) after 30 min, as confirmed through gel electrophoresis. Therefore, a 30-min amplification time was deemed optimal since it provided the longest reaction time without any color change observed in the NTC.

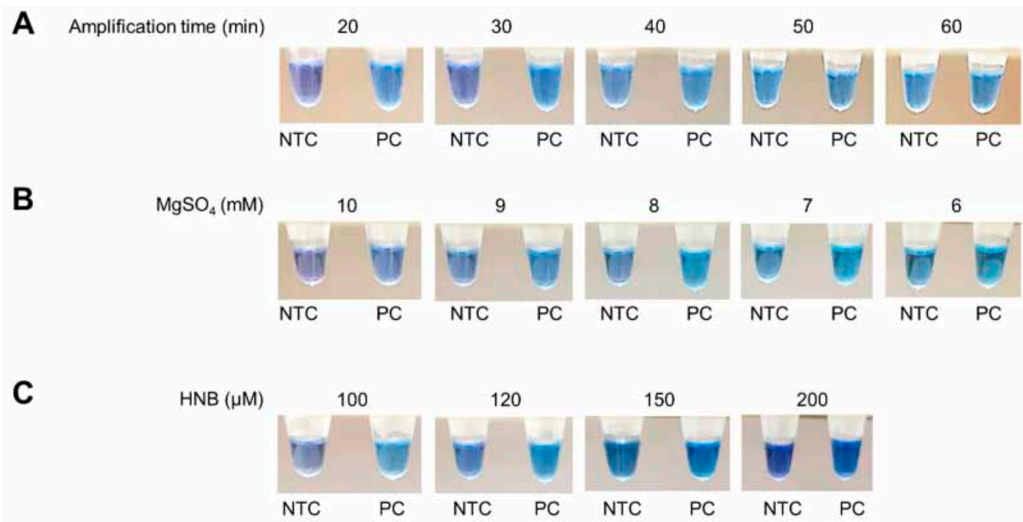

Figure 1. Optimization of RT-LAMP assay based on HNB by varying the amplification time (**A**), $MgSO_4$ concentrations (**B**), and HNB concentrations (**C**), using no template control (NTC) and positive control (PC).

Subsequently, the concentrations of $MgSO_4$ (ranging from 6 to 10 mM) and HNB dye (100, 120, 150, and 200 µM) were assessed at 65 °C for 30 min, followed by inactivation at 80 °C for 10 min. It was observed that a distinct color change from purple (indicating a negative result) to sky blue (indicating a positive result) occurred at 8 mM $MgSO_4$ (Figure 1B) and 120 µM HNB dye (Figure 1C). Therefore, the optimal conditions for the RT-LAMP assay based on HNB were determined as 8 mM $MgSO_4$ and 120 µM HNB with an amplification time of 30 min at a temperature of 65 °C.

3.2. Evaluation of the Developed Colorimetric RT-LAMP Assay Based on HNB in Clinical Samples

To evaluate the developed colorimetric SARS-CoV-2 RT-LAMP assay using HNB, a total of 82 clinical samples collected between May 2020 and December 2021 were used for the detection of SARS-CoV-2 RNA. Representative positive and negative results are shown in Figure 2. Of these samples, 38 samples were tested positive, and 44 samples tested negative by qRT-PCR. Of the 38 positive samples, 35 tested positive with the colorimetric RT-LAMP based on HNB. Three samples that showed false-negative results had CT values of 29.36, 34.35, and 36.32, indicating low SARS-CoV-2 RNA levels in these samples. In this series of clinical samples, the RT-LAMP based on HNB showed a sensitivity for the detection of SARS-CoV-2 of 92.1% (95% CI, 78–98%) and a specificity of 93.2% (95% CI, 81–98%) as compared to the qRT-PCR assay (Table 1).

Table 1. Evaluations of colorimetric RT-LAMP assays based on HNB (92.1% Sensitivity, 95% CI, 78–98%; 93.2% Specificity, 95% CI, 81–98%) and of colorimetric RT-LAMP assays based on cresol red using clinical specimens (80.3% Sensitivity, 95% CI, 69–89%; 97.0% Specificity, 95% CI, 83–100%).

Assays		**RT-LAMP**			
		Based on HNB (N = 82)		**Based on Cresol Red (N = 94)**	
		Positive	**Negative**	**Positive**	**Negative**
qRT-PCR	Positive	35 (92.1%)	3 (7.9%)	49 (80.3%)	12 (19.7%)
	Negative	3 (6.8%)	41 (93.2%)	1 (3.0%)	32 (97.0%)

N, total number of samples tested.

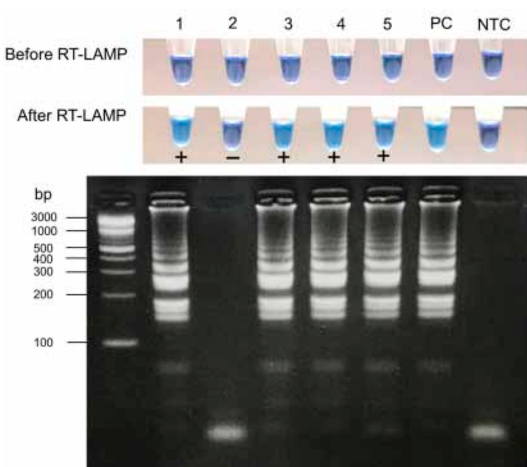

Figure 2. SARS-CoV-2 detection in clinical samples by colorimetric RT-LAMP assay based on HNB. A color change from purple to sky blue was only observed in tubes containing SARS-CoV-2 RNA (no. 1, 3, 4, and 5), whereas the negative sample remained purple (no. 2). The RT-LAMP products have the same gel electrophoresis pattern and correlate with the changes in color. The gel electrophoresis results revealed ladder-like band patterns in SARS-CoV-2 samples but not in negative samples. PC, positive control; NTC, no template control.

3.3. Evaluation of the Developed Colorimetric RT-LAMP Assay Based on Cresol Red in Clinical Samples

The sensitivity and specificity of the colorimetric RT-LAMP based on cresol red for the detection of SARS-CoV-2 RNA were assessed with 94 clinical samples collected between June 2021 and March 2022. Of the 61 samples that tested positive by qRT-PCR, 49 were positive by RT-LAMP based on cresol red, and 12 were negative. Among the 12 false-negative samples, 8 samples had a CT value higher than 35 by the qRT-PCR assay, indicating very low SARS-CoV-2 RNA levels, and 4 samples had a CT value below 35 (i.e., 18.25, 19.84, 21.25 and 21.58). This assay showed a sensitivity of 80.3% (95% CI, 69–89%) and a specificity of 97.0% (95% CI, 83–100%) (Table 1). Gel electrophoresis was also used to confirm the results (Figure 3).

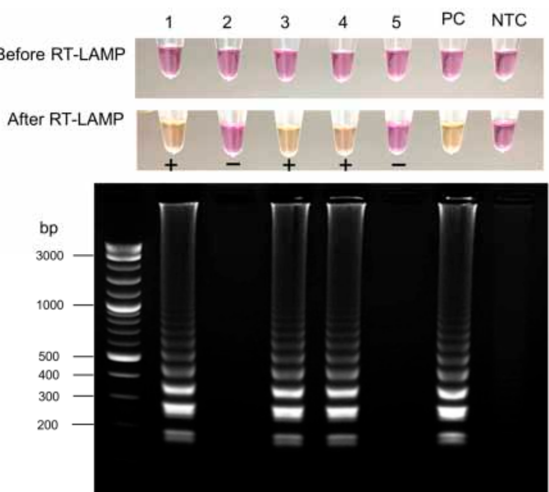

Figure 3. SARS-CoV-2 detection in clinical samples using RT-LAMP with cresol red-based colorimetric detection. A color change from pink to yellow was observed in tubes containing SARS-CoV-2 RNA (no. 1, 3, and 4), while negative samples remained pink (no. 2, and 5). The electrophoresis pattern of the RT-LAMP products is in the same order as the tubes. The results showed the presence of typical ladder-like band patterns in SARS-CoV-2 samples and no ladder-like pattern in negative samples. PC, positive control; NTC, no template control.

4. Discussion

We describe here an RT-LAMP assay for SARS-CoV-2 that can be used with two different colorimetric detections: a metal indicator dye (HNB) and a pH indicator dye (cresol red). This assay offers several advantages over qRT-PCR or other RT-LAMP assays using other reporting platforms such as fluorescence dyes [20–22], commercialized colorimetric LAMP [16], turbidity measurement [11], and lateral flow strips [23,24].

First, our assay is simple to perform. The reaction is carried out in a single, closed tube placed in a simple temperature-controlled device and read with the naked eye, making it suitable for resource-limited settings where access to sophisticated or specialized instruments is limited. Second, the results are obtained within 40 min, which is crucial for making timely decisions and effective management of infected individuals. Third, the cost of the assay is below 15 USD. Furthermore, this assay has acceptable sensitivity and specificity to detect SARS-CoV-2 RNA.

Detection with HNB is favored due to its simplicity, cost-effectiveness, and safety [25]. However, the color transition of HNB may be difficult to distinguish with the naked eye. The color change in cresol red is more easily distinguishable, as it requires only a minimal change in pH level to trigger a color change from pink to yellow [19]. Consequently, the results can be interpreted more readily and confidently with the cresol red indicator. Detection with HNB gave a sensitivity of 92% and a specificity of 93%. Three false-negative results may be due to the high CT value, indicating a low viral load. Detection with cresol red gave a lower sensitivity of 80.3% and a high specificity of 97%. This may be due to the low amount of virus (8 of 12 samples with false-negative results had a CT greater than 35) and/or the different variants circulating during the period of sample collection from June 2021 through March 2022, which may not have been detected. Indeed, mutations in the *N* gene region corresponding to primer binding sites can affect the detection of SARS-CoV-2 RNA [26,27]. However, we cannot verify or refute this hypothesis since the sequencing of these samples could not be done in this study. Furthermore, four samples exhibited false-positive reactions in both RT-LAMP assays, which could potentially be attributed to cross-contamination. The use of multiple primers in the RT-LAMP assay can lead to the production of a large amount of LAMP products, therefore increasing the risk of cross-contamination and false-positive reactions [28]. Overall, the RT-LAMP assay developed in this study revealed sensitivity and specificity comparable to what was reported by Dao Thi VL et al. using different primer sets to evaluate clinical samples [29]. Moreover, RT-LAMP-based HNB assay demonstrated comparable sensitivity and higher specificity when compared to a study from Prakash S et al., where LAMP assay was used to detect SARS-CoV-2 RNA in blood samples [15]. Ultimately, the choice between HNB and cresol red as indicators in the RT-LAMP assay will depend on user preferences, specific requirements, and resource availability. Using a 2-step detection system may be a limitation for a wider use of cresol red-based assay. However, the better discrimination between negative and positive samples makes the results easier to interpret.

This study has some limitations. First, the number of clinical samples tested was relatively low, which may affect the generalizability of the assay's performance. Second, internal control, which could have provided better quality control and detection of potential inhibitory factors, was not included in the assay. Third, the primers used in the study targeted only the *N* gene, which may limit the assay's ability to detect viruses with mutations in the primer regions. However, the *N* gene is highly conserved and abundant. Fourth, the specificity of the developed assay against other related pathogens was not evaluated. Last, an evaluation of the limit of detection of the developed test should be performed. To address these limitations and improve the assay's performance, further studies with larger sample sizes will help establish the robustness and reliability of the assay. Other possible improvements include the addition of an internal control, carrying out a separate reaction with specific primers for *RNaseP*, targeting multiple gene regions to avoid the risk of false negatives if mutations occur in one particular region, and increasing the sensitivity and specificity of the assay.

5. Conclusions

The RT-LAMP assay using HNB and cresol red dye as visual indicators for SARS-CoV-2 detection represents a great promise for rapid and accurate diagnosis. This assay combines the advantages of simplicity, sensitivity, and rapidity, enabling the detection of SARS-CoV-2 in a cost-effective and timely manner. This assay also provides significant potential for addressing the demand for rapid diagnostics and controlling the spread of SARS-CoV-2.

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Conflicts of Interest: The authors declare no conflict of interest.

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