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Original Research Papers

Detection of SARS-CoV-2 from Indonesia with colorimetric saliva-based RT-LAMP method using a thermal cycler

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Abstract
Coronavirus disease 2019 (COVID-19) is an acute respiratory system disorder caused by the SARS-CoV-2 (Severe acute respiratory syndrome coronavirus 2). The gold standard detection method is RT-qPCR (reverse transcription-quantitative polymerase chain reaction). The weaknesses of the RT-qPCR method are the long detection time and nasopharyngeal swab sampling causing discomfort to the patient. An alternative method for detecting the SARS-CoV-2 virus is colorimetric saliva-based RT-LAMP (reverse transcription loop-mediated isothermal amplification). The purpose of this study was to determine the ability of the RT-LAMP saliva based colorimetric method using a thermal cycler as an alternative for detecting SARS-CoV-2 in Indonesia. This study used the Indonesian SARS-CoV-2 primary design. The samples used positive and negative COVID-19 saliva based on the results of the RT-qPCR test from the PKU Muhammadiyah Yogyakarta Hospital. The stages of the research included optimizing the RT-LAMP conditions, and taking saliva samples followed by visualizing the results by colorimetric and electrophoresis. Colorimetric testing was carried out with the addition of phenol red and the color change from pink to yellow was observed. The results showed that the RT-LAMP could run optimally at 65°C for 45 minutes. The colorimetric RT-LAMP test in this study was ineffective because it couldn't detect positive samples compared to electrophoresis results. Factors causing this included the small number of amplicons, incompatible RT-LAMP reagents used for the colorimetric method using phenol red, and phenol red which cannot work optimally, requiring more suitable pH indicator. Further research needs to be carried out by replacing other visualization methods.

Keywords: colorimetric; COVID-19; RT-LAMP; saliva; thermal cycler

1. Introduction

Coronavirus disease 2019 (COVID-19) is an acute respiratory system disorder caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) virus (WHO, 2020). The SARS-CoV-2 virus was first discovered in Wuhan City, China, in late December 2019 and spread to countries worldwide. Typical clinical symptoms of COVID-19 patients are fever, dry cough, difficulty breathing, headache, and pneumonia (Wyllie et al., 2020). As of August 2, 2022, the number of positive confirmed cases of COVID-19 in the world has amounted to 572,239,451, with a death rate of 6,390,401. Meanwhile, the number of positive confirmed cases of COVID-19 in Indonesia is 6,210,794, and the death rate is 157,004 (COVID Task Force, 2022). Coronavirus is a positive single-stranded RNA virus. SARS-CoV-2 has a genome size of about 30 kb (Wu et al., 2022). Transmission of the SARS-CoV-2 virus can occur through droplets when a person comes into close contact within 1 meter with an individual experiencing respiratory symptoms such as coughing or sneezing. One of the transmission media of SARS-CoV-2 is salivary droplets produced by breathing, talking, and sneezing (Guna, 2020).

Since its appearance, SARS-CoV-2 has continued to undergo mutations. Based on the increase in transmission, virulence, and the ability to avoid immunity formed from vaccination, several variants are classified as variants of concern (VOCs), including the alpha variant (Iacobucci, 2021), the beta variant (Tegally et al., 2020), the gamma variant (Faria et al., 2021), the delta variant (Wall et al., 2021), and the omicron variant (Torjesen, 2021). The mutations produced by the SARS-CoV-2 virus make its transmission faster, so it requires a fast detection method to prevent the transmission of the variant. The standard gold method for the detection of SARS-CoV-2 is RT-qPCR. However, this method takes 24 hours until the results of diagnosis and sampling are released through a nasopharyngeal swab that causes discomfort and can potentially cause cross-infection of medical personnel (Leber et al., 2021; Wyllie et al., 2020). Alternative samples that can be used for the detection of SARS-CoV-2 are saliva (Xu et al., 2020). Saliva samples have often been used before to detect the presence of viruses that cause infectious diseases such as HIV, Ebola, and rabies (Corstjens et al., 2016).

Loop-mediated Isothermal Amplification (LAMP) is a rapid DNA amplification technology applied to detect pathogens such as viruses, bacteria, and malaria (Thai et al., 2004). The LAMP method uses 4 or 6 primers to bind to six target DNA regions and has high specificity. The LAMP reaction generally takes place at a constant temperature of 65°C, and the target DNA can be amplified within 30 minutes, so the tool used with this method is more straightforward. The development of the LAMP method is the Reverse Transcription Loop-mediated Isothermal Amplification (RT-LAMP) method.

RT-LAMP products can be analyzed through various means, such as conventional DNA-intercalating dyes, agarose gel electrophoresis, real-time fluorescence, and luminescence. In addition, RT-LAMP test results can be observed with color changes (colorimetry) using pH indicators such as phenol red, cresol red, neutral red, and m-cresol purple (Tanner et al., 2015). This is because pyrophosphates and protons are produced during the DNA polymerization process after the incorporation of deoxynucleotide triphosphates (Amaral et al., 2021). RT-LAMP is colorimetrically a detection method that does not require expensive tools to observe results and faster observation times. Therefore, this study aims to determine the ability of the RT-LAMP colorimetric method as an alternative to covid-19 detection in Indonesia.

2. Research Methods

This research uses experimental methods that are carried out on a laboratory scale. This test was carried out using saliva samples from positive and negative COVID-19 patients from PKU Muhammadiyah Hospital, Yogyakarta City.

2.1. RT-LAMP Primer Preparation

This study uses the Indonesian SARS-CoV-2 primer design. Primary preparation begins with the manufacture of 100µM primary stock. The composition of the primary dilution of 50x the reaction can be seen in Table 1. Each primer is inserted into a microtube, then a 90µl TE buffer and vortex are added. Stocks of primers and ready-mix primers are stored in freezers.

Table 1. The composition of the primary dilution of 50x the reaction

Prime	Concentration per Reaction	Volume per Reaction
F3	2.0µM	2.5µl
B3	2.0µM	2.5µl
FIP	8.0µM	10.0µl
BIP	8.0µM	10.0µl
LoopF	4.0µM	5.0µl
LoopB	4.0µM	5.0µl

2.2. RT-LAMP Temperature and Time Optimization

RT-LAMP optimization uses positive control of the SARS-CoV-2 RdRp gene with a concentration of 0.1ng. 15µl RT Isothermal Master Mix ISO-DR004-RT100 (Opti Gene) reagent, 2.5µl primer mix, and 2.5µl NFW, inserted into a microtube and then vortex. The reagent was transferred to the PCR tube, and then a positive control of the RdRp gene was added by five µl and then spin down it. Furthermore, the RT-LAMP test was carried out using a thermal cycler at a temperature of 65°C with a time variation of 10 minutes, 20 minutes, 30 minutes, 45 minutes, and 60 minutes. After that, visualize the test results using electrophoresis.

2.3. Saliva Sampling

Saliva sampling will be carried out from 12–17 September 2022. The saliva sampling technique in this study was purposive sampling. The samples used are patients who have received COVID-19 detection results based on the RT-qPCR test. The number of saliva samples obtained was three positive and two negative COVID-19 examples. Before sampling, the patient fills in the informed consent that has been prepared. Selection is made using the patient being asked not to eat or drink for 30 minutes and then spitting saliva in a sterile urine cup (Landry et al., 2020). After that, the saliva sample is stored in the refrigerator at 4°C while preparing for the heat inactivation stage.

2.4. Heat inactivation Saliva Samples

Heat inactivation of saliva samples is carried out directly on the same day after sampling. Saliva samples were taken from the refrigerator and diluted using a sterile Phosphate buffered saline (PBS) solution with a concentration of 1:2 (Matic et al., 2021). Saliva samples were taken as much as 0.2 mL using sterile syringes. Then it is inserted into a microtube containing a 0.4mL PBS solution. Then heat inactivation is carried out using a heating block at a temperature of 95°C for 10 minutes (Yang et al., 2021). Furthermore, saliva samples were stored at -80°C inside the freezer.

2.5. RT-LAMP Test

Master Mix Reagent of 15µl, primer mix of 2.5µl, and NFW of 2.5µl are inserted into micro tubes and then vortexed. Then the reagent was transferred into the PCR tube, and 5µl of positive and negative saliva samples of COVID-19 were added and then spin down it. Furthermore, the test was carried out using a thermal cycler at a temperature of 65°C for 45 minutes.

2.6. Visualization of Results

This study uses colorimetric visualization methods and electrophoresis. Colorimetric visualization of results is carried out using a pH indicator, namely phenol red. The amplified PCR tube added phenol red by 1µl (He et al., 2020). Then a discoloration was observed on the tube containing the saliva sample—the change in pink shows positive results to yellow (Huang et al., 2020).

Electrophoresis is carried out using 2% agarose gel. The agarose gel is inserted into the electrophoresis tank with the good side at (-), and then the TBE buffer is added to cover the gel. Furthermore, markers (100bp), positive control, non-template control (NTC), and positive and negative salivary samples of 5µl were inserted sequentially into the well. The electrophoresis process is carried out with an electrical force of 100 volts for 30 minutes, then the emerging DNA band is observed with a UV transilluminator.

3. Results and Discussion

3.1. RT-LAMP Optimization Electrophoresis Results

RT-LAMP is a very efficient examination technique in conducting DNA amplification. According to Feranisa (2016), this method has high sensitivity and specificity and faster testing times. Detection of SARS CoV-2 using the RT-LAMP method using a primer that functions to amplify certain areas requires temperature and time optimization to obtain optimal RT-LAMP conditions. Optimization of RT-LAMP conditions on thermal cyclers is carried out at a temperature of 65°C with time variations of 10 minutes, 20 minutes, 30 minutes, 45 minutes, and 60 minutes. The selection of 65°C temperature follows the optimum temperature recommendations of the kit used in this study. The time variation at this stage aims to get the optimal time in the RT-LAMP test. The results of the RT-LAMP optimization electrophoresis in this study can be seen in Figure 1.

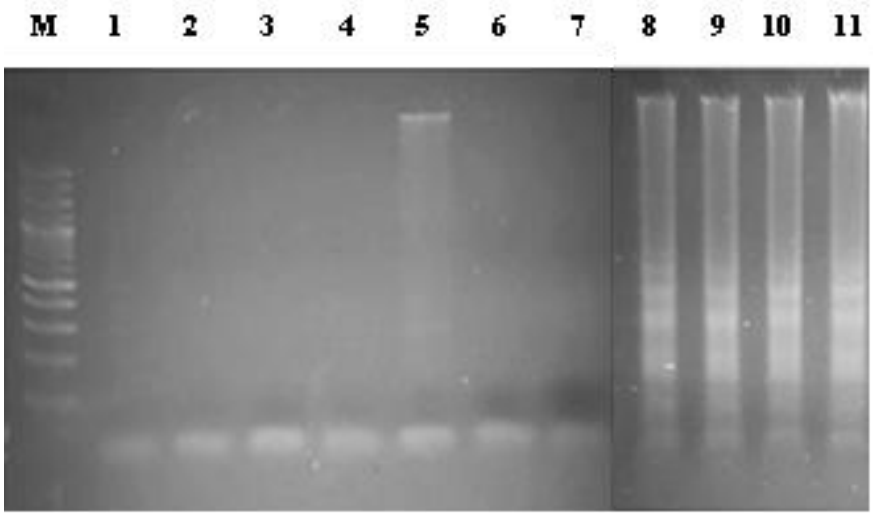

Figure 1. Rt-LAMP optimization electrophoresis results M (Marker), 1 (10 minutes replay 1), 2 (10 minutes replay 2), 3 (20 minutes replay 1), 4 (20 minutes replay 2), 5 (30 minutes replay 1), 6 (30 minutes replay 2), 7 (NTC), 8 (45 minutes replay 1), 9 (45 minutes replay 2), 10 (60 minutes replay 1), 11 (60 minutes replay 2).

The RT-LAMP optimization electrophoresis results showed that DNA bands looked thin at 30 minutes of replay one and almost invisible at 30 minutes of replay 2. At 45 minutes and 60 minutes, evident DNA bands were visible. In non-template control (NTC), there are no DNA bands. The appearance of DNA bands is a sign that the sample can be amplified at that time.

Based on the optimization results, it can be stated that the optimal condition of RT-LAMP using a thermal cycler is at a temperature of 65°C for 45 minutes. This timing is based on electrophoresis results that show the DNA band is very clearly visible within 45 minutes, while at 30 minutes, the sample amplification is not optimal because the DNA band that appears looks thin. At 60 minutes, it takes a long time for the RT-LAMP test. The results of this optimization follow research by Li et al. (2021), which shows that the RT-LAMP test using a thermal cycler can be carried out at a temperature of 65°C for 45 minutes.

3.2. Colorimetric Saliva-Based RT-LAMP Test Results

The colorimetric test in this study used a pH indicator, namely phenol red, which functions as an indicator of color change to determine the presence of the SARS-CoV-2 virus in the sample. The results of the colorimetric saliva-based RT-LAMP test can be seen in Figure 2

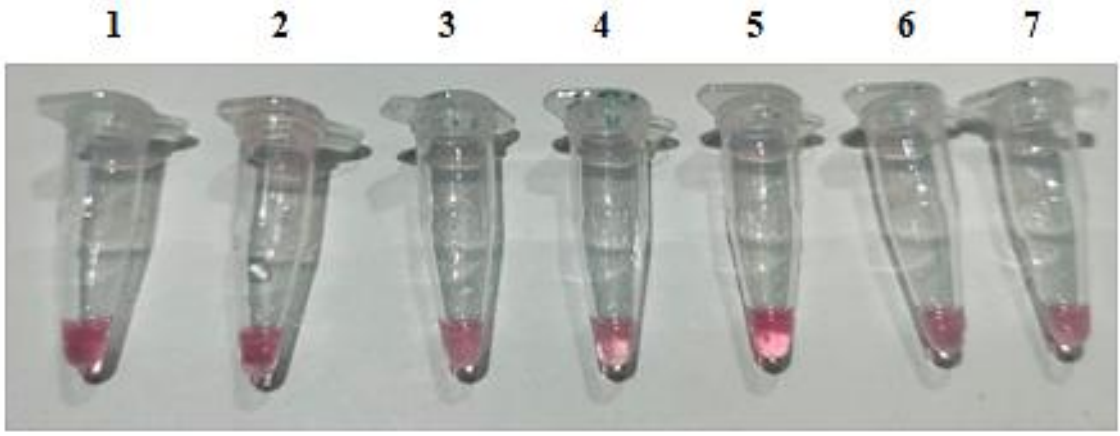

Figure 2. Colorimetric saliva-based test results RT-LAMP 1 (Positive control), 2 (NTC), 3-5 (Positive saliva sample), 6-7 (Negative saliva sample).

Saliva samples obtained from participants were diluted using PBS and then heat-inactivated at 95°C for 10 minutes. The salivary sample heat inactivation treatment aims to inactivate the SARS-CoV-2 virus so that it is not transmitted to researchers or health workers during the testing process. After heat inactivation, saliva samples are stored at -80°C to maintain the quality of the sample from being damaged. Heating at 95°C when heat inactivation can reduce the inhibitor power caused by inhibitors in salivary samples, thereby facilitating the RT-LAMP test process (Morais et al., 2022). Inhibitors in salivary samples are antibodies and enzymes that can affect viral RNA instability and inhibit the detection process (Griesemer et al., 2021).

The results of the RT-LAMP watery metric saliva-based test in Figure 2 show that there is no discoloration on all tubes. The pink color indicates that there is no SARS-CoV-2 virus in the sample, while at the time of the RT-LAMP test, tubes 3,4 and 5 contain positive saliva containing the SARS-CoV-2 virus, and there should be a change in pink color to yellow. Based on these results, it can be stated that a false negative occurred. The factors that caused the occurrence of false negatives in this study were the number of amplicons that may be less and the CT (cycle threshold) value of more than 30. According to Aoki et al. (2021), the RT-LAMP colorimetric test correlates with the patient's Ct value. The results of the RT-LAMP colorimetric test in patients who had a CT of more than 30, when observed directly with the naked eye, showed false negative and indeterminate results.

Different RT-LAMP reagents, such as Master Mix, can also cause different results in this study from previous studies. This study uses RT Isothermal Master Mix ISO-DR004-RT100 products from OptiGene. Previous studies conducted by Yu et al. (2020), Janíková et al. (2021), Lalli et al. (2021), Reynés et al. (2021), and dos Santos et al. (2021) used WarmStart Colorimetric LAMP 2X Master Mix (New England Biolabs), while Huang et al. (2020) used WarmStart Colorimetric LAMP 29 (New England Biolabs).

In addition, the phenol red used in this study has been stored for a long time, so there is a possibility of damage that causes no discoloration in the samples tested. The types of pH indicators that can be used other than phenol red are hydroxy naphthol blue (HNB), xylenol orange (XO), and lavender green (LG) (Jaroenram et al., 2022). Based on the results of the colorimetric test in this study which showed a false negative, it is necessary to confirm it by electrophoresis.

3.3. Saliva-Based RT-LAMP Electrophoresis Results

The results of RT-LAMP-based saliva electrophoresis in this study can be seen in Figure 3.

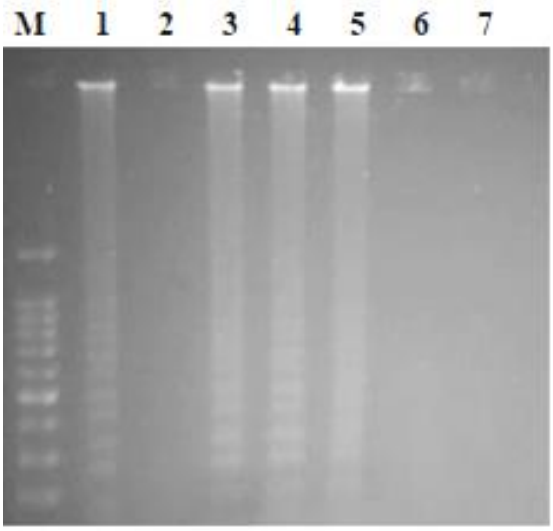

Figure 3. Saliva electrophoresis results based RT-LAMP M (Marker), 1 (Positive control), 2 (NTC), 3-5 (Positive saliva sample), 6-7 (Negative saliva sample)

The electrophoresis results for 30 minutes in Figure 3 showed that in salivary samples, DNA bands appeared in positive controls (1) and positive saliva samples (3-5). NTC (2) and negative salivary samples (6-7) had no DNA bands. The results of this electrophoresis are in accordance with the proper research results. This result further strengthens that there is a false negative in the RT-LAMP colorimetric test results with the red phenol indicator.

4. Conclusion

Based on the research results, it can be concluded that the colorimetric saliva-based RT-LAMP method using thermal cyclers in this study has not been able to detect SARS-CoV-2. Nonetheless, the electrophoresis results showed the presence of DNA bands in the positive sample. This indicates that SARS-CoV-2 DNA amplification occurred via RT-LAMP at 65°C for 45 minutes.

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