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ORIGINAL RESEARCH

The Performance of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) Virus Using the Colorimetric Reverse-Transcription Loop Mediated Isothermal Amplification (RT-LAMP) Method in Saliva Specimens of Suspected COVID-19 Patients

Dewi Kartika Turbawaty, Andy Sudjadi, Leni Lismayanti, Tiene Rostini, Verina Logito

Department of Clinical Pathology, Faculty of Medicine Padjadjaran University/ Dr. Hasan Sadikin General Hospital, Bandung, West Java, Indonesia

Correspondence: Dewi Kartika Turbawaty, Email dewi.kartika@unpad.ac.id

Introduction: Corona Virus Disease-19 (COVID-19) is a disease caused by Severe-Acute-Respiratory-Syndrome-Coronavirus-2 (SARS-CoV-2). The most reliable and widely accepted method for diagnosing this infection, despite facing various challenges, is the Reverse Transcription Polymerase Chain Reaction (RT-PCR) method, which utilizes nasopharyngeal swab sample. Reverse-transcription loop mediated isothermal amplification (RT-LAMP) is a simpler nucleic acid amplification method compared to the RT-PCR method. This method has several advantages, including: of amplification at constant temperature, faster results, and potentially greater examination capacity.

Purpose: This study aimed to compare the validity of the RT-LAMP method using saliva specimens with that of the RT-PCR method using nasopharyngeal smears.

Methods: This was an analytical observational study with a cross-sectional design. The participants were inpatients in the COVID-19 special isolation building of Hasan Sadikin General Hospital, Indonesia with a probable (clinical symptoms of covid, but not confirm NAAT examination) or confirmed diagnosis of COVID-19 from September 2021 to February 2022. The inclusion criteria are COVID-19 patients with symptoms, adult subjects, and composite mentions. Patients who were unable to secrete saliva were also excluded.

Results: In total, 118 specimens were collected. The validity test results of the saliva specimens using the RT-LAMP method showed sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), of 65.5%, 100%, 100%, and 75%, respectively. The results increased in subjects treated between 3 and 7 days after symptom onset ie 73.2%, 100%, 100%, and 82.3%, respectively.

Conclusion: The very strong specificity accompanied by good sensitivity and NPV in the group of subjects treated 3–7 days after the onset of symptoms indicates that the RT-LAMP method using saliva specimens can be an efficient and reliable alternative tool in detecting the SARS-CoV-2 virus.

Keywords: COVID-19, SARS-CoV-2, RT-LAMP, RT-PCR, saliva

Introduction

The first case of *Coronavirus Disease* (COVID-19) was found in Wuhan City, China in December 2019. This disease is caused by the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), and it affected many countries. On March 11, 2020, it was declared a pandemic by the World Health Organization (WHO). The rapid spread of COVID-19 globally is attributable to its long incubation period, high transmission rate, and asymptomatic status among carriers. The

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virus is mainly transmitted between people through contact routes and respiratory droplets. Indonesia is the fourth most populated country in the world to be affected by the COVID-19 pandemic, and various areas of national concern have been impacted, including economics, politics, and human welfare. The first positive COVID-19 case in Indonesia was confirmed on 2 March 2020. Since then, several COVID-19 outbreaks have been reported in various regions of Indonesia. As of 4,763,252 cases were reported in Indonesia, as at February 13, 2022, with a mortality rate of 145,065.[1,2]

One of the strategies most widely used to control the spread of COVID-19 in Indonesia is laboratory examination but there are still many remote areas in Indonesia where it is difficult to meet the facility standards required for laboratory examination. The gold standard for diagnosing SARS-CoV-2 is reverse transcription polymerase chain reaction (RT-PCR) testing. Specimens used for this test are obtained from the upper respiratory tract, most commonly nasopharyngeal smears. However, the use of nasopharyngeal smears to detect SARS-CoV-2 has various challenges, including complex examination methods, high risk of transmission to health workers, and the need for personal protective equipment (PPE).[3,4]

The use of saliva specimens have been widely studied for the detection of SARS-CoV-2 because of their various advantages over nasopharyngeal smears. These advantages include non-invasiveness, the ability to be collected by patients without the help of health workers, reduction in the requirement for operational tools in performing nasopharyngeal smears, and the need for PPE. However, the sensitivity of saliva specimens for detecting SARS-CoV-2 varies from 55% to 85%. A study conducted by Kelvin in Hong Kong in 2020 showed that out of 12 patients with confirmed SARS-CoV-2 infection based on RT-PCR results from nasopharyngeal smear specimens, SARS-CoV-2 was detected in 11 patients with saliva specimens using viral culture examination. It was concluded that the viral load resulting from saliva specimens is directly proportional to the severity of the disease. Other studies have shown that the viral load in saliva decreases after treatment.[5–11]

Reverse-transcription loop-mediated isothermal amplification (RT-LAMP) is a nucleic acid amplification method used to diagnose diseases and detect microorganisms. This method is simpler than RT-PCR using nasopharyngeal smear specimens which requires complex facilities and trained health workers. In addition, RT-PCR has a limited capacity for a small number of examinations, resulting in a delayed result. Isothermal PCR, such as RT-LAMP, is a potential alternative method to overcome the limitations of RT-PCR. The RT-LAMP method has several advantages, such as performing amplification at a constant temperature of approximately 65 °C, eliminating the need for a thermal cycler, faster test results in less than 30 min, and potential for greater diagnostic testing capacity. It also maintains the expected sensitivity and specificity values equivalent to those of RT-PCR.[3,12,13] Therefore, this study aimed to analyze the validity of the detection of SARS-CoV-2 using the RT-LAMP method on saliva specimens compared with the gold standard RT-PCR method using nasopharyngeal smear specimens.

Material and Methods

Study Population

The participants of this study were patients hospitalized in the COVID-19 special isolation building of Hasan Sadikin General Hospital Bandung, diagnosed with either a probable or confirmed case, following the guidelines outlined in the Decree of the Minister of Health of the Republic of Indonesia No. HK.01.07/MENKES/4641/2021. This study was conducted between September 2021 and February 2022. The inclusion criteria were probable or confirmed COVID-19 with symptoms of severity listed in the guidebook, adult subjects (aged >18 years), and patients in a state of composition. Exclusion criteria are patients who are unable to secrete saliva at the same time or maximum of 24 hours after taking the nasopharyngeal smear specimens, the volume of saliva was less than 0.5 mL, and the specimens were the sputum and colored.

Specimen Collection

The participants will be required to collect saliva at a designated location. The study included the collection of nasopharyngeal and saliva specimens on the first or second day of admission to the hospital from September 1, 2021, to February 28, 2022. Normal saliva specimens that naturally accumulate in the mouth without coughing or gargling before collection are required. The participants were instructed to refrain from eating, drinking, and smoking for 1 h before the collection of saliva and nasopharyngeal specimens. It is stated on the kit insert to prevent pH and food residue from affecting the examination results. The collected saliva using a sterile container with a screw cap was stored in a refrigerator at a temperature–2-8°C in the molecular biology laboratory of Hasan Sadikin General Hospital for

a maximum of 48 h before SARS-CoV-2 detection using the RT-LAMP method. The RT-LAMP method can detect as low as 50 copies of virus in 1 µL of saliva, and has Sensitivity 92.6% and Specificity 93.3%. Saliva was collected on the same day as the nasopharyngeal swab, or within a maximum of 24 h. Repeated saliva collection should be performed when necessary.

Laboratory Procedure

For the virus inactivation process, sample inactivation using heat was performed by incubating the specimens at 65°C for 15 min. For pre treatment sample, prepare the specimen and placed in a Biological Safety Cabinet (BSC). When the specimen was frozen, it was thawed completely at room temperature and homogenized. Fifty microliters of Pre-treatment Reagent and 50 µL of saliva were added to a 1.5 mL tube, mixed until homogeneous using a vortex. The homogenized samples were incubated at 95°C for 20 min.[14]

For the RT-LAMP Process, two Loop-Mediated Isothermal Amplification (LAMP) mixes were prepared for each specimen, namely, IC LAMP Mix and N Gene LAMP Mix. The IC LAMP Mix and aliquot 13 µL to each PCR tube. Next, 7 µL of extracted RNA was added to each PCR tube containing the IC LAMP Mix. The extracted RNA may have contained sediment at the bottom of the tube. Be cautious not to include the sediment and only to add the supernatant to the IC LAMP Mix. Then, 7 µL of each Internal and Negative Control were added to the respective PCR tubes. The mixture was vortexed and centrifuged for a few seconds to remove air bubbles and bring the mixture fluid to the bottom of the tube. The color of the reaction mixture was observed after the addition of the sample. The mixture was incubated at 65°C for 30 min.[14]

For the RT-LAMP assay, the color of the reaction mixture should be observed before and after the RT-LAMP incubation process to obtain initial and final color information. The color of the mixture before the RT-LAMP reaction was pink. The expected final color of the specimens containing SARS-CoV-2 RNA was yellow or orange-based, whereas those lacking SARS-CoV-2 RNA were pink or red. Some RT-LAMP reactions may not produce optimal changes, resulting in ambiguous colors. In this case, the RT-LAMP procedure was repeated by adjusting the pH of the specimen using adjustment reagents or 0.061 N HCl according to the instrument guidelines. The color of the specimen is determined using the "Color Grab" application, which can be downloaded on smartphones.[14]

Data Analysis

The sample size was determined using a formula for diagnostic tests, and a minimum sample size of 106 participants was obtained. To anticipate data loss, the sample size was increased by 10%, resulting in 117 participants. This was an analytical, observational study with a cross-sectional design. A diagnostic test was used to assess the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of SARS-CoV-2 using the RT-LAMP method on saliva specimens to detect SARS-CoV-2. This study was approved by the Ethical Committee of the Faculty of Medicine, Padjadjaran University and Dr. Hasan Sadikin General Hospital (No: LB.02.01/X.6.5/180/2021.

Results

A total of 124 participants were screened, and six specimens were excluded from the study because 1) the patient provided a specimen in the form of sputum despite repeated collection, 2) the subject was unable to produce saliva, and 3) the subject produced colored saliva. The exclusion of these subjects was based on the observation that they provided consistent saliva specimens despite repeated collection. Participants' characteristics are presented in Table 1.

Table 2. shows RT-LAMP method with saliva specimen to detect SARS-CoV-2 compared to RT-PCR method with nasopharyngeal smear specimen as the gold standard.

The number of specimens used in this study was 118, consisting of 58 nasopharyngeal smears positive by RT-PCR and 60 negative by RT-PCR.

There were 60 true negative subjects with negative RT-PCR and RT-LAMP results from subjects with a Ct of ≤ 40 (RT-PCR NPS).[15] We also included 38 true-positive subjects with positive RT-PCR and RT-LAMP results. A total of 20 subjects showed negative RT-LAMP and positive RT-PCR results, resulting in false-negative results. The study did not show any subjects with positive RT-LAMP or negative RT-PCR results. Therefore, no false-positive results were obtained, ensuring the validity of the saliva specimen RT-LAMP method in comparison to the RT-PCR method, with a Ct value of ≤ 40.

Table 1 Characteristics of Study Subjects

Characteristics of Patients	Total N (%)
Age (years)	
18–25	23 (19.5)
26–35	23 (19.5)
41–45	15 (12.7)
46–55	17 (14.4)
56–65	28 (23.7)
> 65	12 (10.2)
Gender:	
Male	52 (44.1)
Female	66 (55.9)
Onset of Clinical Symptom Before Treated (days)	
<3	21 (17.8)
3–7	92 (78.0)
> 7	5 (4.2)

Table 2 Validity Test of RT-LAMP Method Saliva Specimen

	RT-LAMP saliva	RT-PCR Positive	NPS Negative	Total	Sn(%)	Sp(%)	PPV(%)	NPV(%)	Youden's index
All Subjects (n=118)	Positive	38	0	38					
	Negative	20	60	80	65,5	100	100	75	0,655
	Total	58	60	118					
Subject admitted 3–7 days After Symptom Onset (n=92)	Positive	30	0	30					
	Negative	11	51	62	73,2	100	100	82,3	0,732
	Total	41	51	92					
Subjects with Ct<20 (n=92)	Positive	15	12	27					
	Negative	2	63	65	88,2	84	55,6	96,9	0,722
	Total	17	75	92					

Abbreviations: RT-LAMP saliva, reverse-transcription loop mediated isothermal amplification from saliva specimen; RT-PCR NPS, reverse transcription polymerase chain reaction from nasopharyngeal smear; Sn, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.

A validity test was also conducted based on the length of symptom onset until the patient was hospitalized, with 92 subjects admitted 3–7 days after symptom onset (78%). Furthermore, 51 true negative subjects were confirmed by negative outcomes using both the RT-PCR and RT-LAMP methods. Thirty subjects had true positive results, as shown by positive outcomes in both saliva RT-LAMP and nasopharyngeal smear RT-PCR methods, with a Ct value range of 15.96 to 29.75. Eleven subjects in this study obtained false-negative results, and none obtained false-positive results.

The validity test was carried out on subjects who were treated 3–7 days after symptom onset, with a Ct≤ value of 20. There were 63 true-negative and 15 true-positive results. Furthermore, 2 subjects in this study obtained false-negative results, and 12 had false-positive results.

Discussion

To the best of our knowledge, this study is the first in Indonesia to examine the validity of the RT-LAMP method using saliva specimens compared with the gold standard RT-PCR method using nasopharyngeal smear specimens to detect SARS-CoV-2.

In this study, COVID-19 was more common in women age group–55-65 years. Advanced age is the main risk factor for COVID-19 patients with be hospitalized with a high mortality rate. This is because of immunosenescence and comorbid diseases in elderly patients. Generally, elderly patients are more difficult to educate than younger patients are. In this study, participants were informed of the specific saliva collection requirements during the process. The collected saliva must not be sputum, and the subjects should not eat, drink, or smoke 1 h before specimen collection. The education process of each person has a different level of difficulty, which is usually influenced by various factors such as age,

education level, and socioeconomic factors. Therefore, the difficulty in the education process in elderly subjects is a challenge in saliva specimen collection and may have affected the results of the RTLAMP method in this study.[16]

RT-LAMP has attracted attention as a cost-effective molecular testing method for SARS-CoV-2. This study achieved 100% specificity for subjects with negative PCR results. Specificity indicates the ability of a test to declare a negative population as not sick. This is one of the key parameters for validating the diagnostic tests. These results are consistent with those reported by Lu in a study conducted in China in 2022, which showed 100% specificity of the RTLAMP method for saliva specimens was 100%. The very strong specificity shows that the RT-LAMP method using saliva specimens can be an efficient and reliable tool for detecting SARS-CoV 2 virus.[5]

In this study, the sensitivity of the RT-LAMP assay using saliva specimens to diagnose COVID-19 was 65.5%. Therefore, the RT-LAMP method for saliva specimens cannot be used as a screening tool for COVID-19. This method has lower sensitivity than RT-PCR. The 2022 study of Pu in China showed that RT-LAMP using nasopharyngeal smear specimens had a slightly lower sensitivity (92%) than RT-PCR (96%). This low sensitivity occurs because standardized standards for both the type of saliva to be used and the procedure for the RT-LAMP method still do not exist. Based on the type of saliva, posterior oropharyngeal saliva (POPS) specimens were known to have higher sensitivity compared to others, namely spit and drooling saliva. However, POPS specimens cannot be used because of their viscosity, which makes them difficult to extract using a pipette. There is no standardized RNA extraction procedure for RNA amplification using the RT-LAMP method. In this study, the extraction was performed by heating the specimen in a water bath at 95° C for 20 min. This high heating temperature aims to break the virus particles and release RNA, as well as denature and partially inactivate ribonuclease (RNase). Ribonucleases catalyze the degradation of RNA into smaller components, thereby decreasing the sensitivity of the RT-LAMP method to decrease when RNase activity is not deactivated.

The sensitivity of the SARS-CoV-2 detection test using the RT-LAMP method with saliva specimens increased to 88.2% among subjects treated 3–7 days after symptom onset with a Ct value of≤ 20. This increase occurred because the greater the SARS-CoV-2 *viral load* in the specimen, the more the viral RNA was released.

A 2021 study by Anahtar in America showed that the addition of *Tris(2- carboxyethyl) phosphine* (TCEP) buffer or *divalent cation chelator ethylenediaminetetraacetic* acid (EDTA) after heating at 95° C is effectively lysed the virus and inactivated RNase. In this case, TCEP and EDTA increased the sensitivity from 59% to 87.5%. TCEP is a reducing reagent that inactivates RNase activity through the reduction of disulfide bonds present in the enzyme. In contrast, EDTA can bind the divalent cations necessary for nuclease activity. In this study, no additional *buffer in* the extraction. Therefore, RNase activity was not completely deactivated, and the RNA released from the virus was denatured by RNase and was not detected using the RT-LAMP m*et*hod.[7,17,18]

In addition to sensitivity and specificity, the NPV and PPV were calculated to determine the usefulness of the RT-LAMP method with saliva specimens in detecting SARS-CoV-2. The NPV of the RTLAMP method was 75%, and the NPV was the proportion of patients who tested negative and were not really sick. This result is consistent with that reported by Alvarez, who obtained an NPV of 75% using the RT-LAMP method. When the RT-LAMP method results are negative, there is still the possibility of a subject obtaining a false-negative result of 25%. NPV increased in subjects treated 3–7 days after symptom onset with Ct≤ 20 from 82.3% to 96.5%.[19]

PPV value was 100%, falling in the category of ”very strong”. PPV was measured as the proportion of patients who tested positive and were actually sick. Both PPV and specificity values are important for estimating the possibility of false positives. The PPV and specificity of the RT-LAMP method for saliva specimens were 100%, indicating that there were no subjects with false positive results. This result is consistent with the report by Iqbal, a study conducted in Sri Lanka in 2022, which obtained a PPV of 100%.[6,20,21]

The RT-LAMP method in the group of subjects treated 3–7 days after symptom onset had 73.2% sensitivity and 82.3% NPV compared to the RT-LAMP method of saliva specimens in all subjects. This occurred because subjects treated 3–7 days after symptom onset had a high SARS-CoV-2 viral load, thereby increasing the sensitivity and PPV of the RT-LAMP method. The increase in sensitivity and NPV while maintaining specificity and PPV values at 100% shows that the RT-LAMP method using saliva specimens is better used on the 3rd to 7th day after symptom onset.

A limitation of this study is that the color produced by the colorimetric RTLAMP reaction was still read manually. Therefore, it has an element of subjectivity even though it has been used in colorgrab applications. Another limitation of this study was the difficulty of saliva collection education in the subjects, specifically the non cooperative patient.

Conclusion and Suggestion

In conclusion, the very strong specificity accompanied by good sensitivity and NPV in the group of subjects treated 3–7 days after the onset of symptoms indicates that the RT-LAMP method using saliva specimens can be an efficient and reliable alternative tool in detecting the SARS-CoV 2 virus. It can help reduce the spread of Covid 19 because the sampling procedures are easier and more comfortable, especially for children and the elderly so the examination costs are cheaper. It can be used for smaller health facilities in Indonesia because the sample collection does not require health personnel so patients can collect their own samples.

Abbreviations

RT-LAMP, Reverse-Transcription Loop-Mediated Isothermal Amplification; RT-PCR, Reverse Transcription Polymerase Chain Reaction; COVID-19, Corona Virus Disease-19; SARS-CoV-2, Severe-Acute-Respiratory-Syndrome-Coronavirus-2; LAMP, Loop-Mediated Isothermal Amplification; NPP, positive predictive value; NPN, negative predictive value; WHO, World Health Organization; PPE, personal protective equipment; POPS, posterior oropharyngeal saliva specimens; RNA, ribonucleic acid; RNase, ribonuclease; TCEP, *Tris(2- carboxyethyl) phosphine*; EDTA, Ethylene diaminetetraacetic acid.

Ethics Approval and Consent to Participate

This study was approved by the Ethical Committee of the Faculty of Medicine, Padjadjaran University and Dr. Hasan Sadikin General Hospital (No: LB.02.01/X.6.5/180/2021). Written informed consent was obtained from all participants. All procedures were performed in accordance with the ethical standards of the Helsinki Declaration of 1975, revised in 2000. All patients provided informed consent to participate in the study, which was approved by the local ethics committee of the Faculty of Medicine Universitas Padjadjaran/Dr. Hasan Sadikin General Hospital, Bandung, Indonesia.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work. Dewi Kartika Turbawaty, Andy Sudjadi, Leni Lismayanti, and Tiene Rostini had full access to all the data in the study and took responsibility for the integrity of the data and accuracy of the data analysis. Dewi Kartika Turbawaty and Andy Sudjadi were the primary authors, and Dewi Kartika Turbawaty was a major contributor to the writing of the manuscript. All the authors have read and approved the final manuscript.

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Disclosure

Dewi Kartika Turbawaty is an academic author, a clinical pathologist, and the Head of Clinical Pathology Department, staff of the Microbiology and Biomolecular Department, Faculty of Medicine Padjadjaran University, and Dr. Hasan Sadikin General Hospital. Andy Sudjadi is a clinical pathologist. Leni Lismayanti is a clinical pathologist and staff member of the Hematology Department, Faculty of Medicine, Padjadjaran University and Dr. Hasan Sadikin General Hospital. Tiene Rostini is a clinical pathologist and staff member in the Clinical Chemistry Department, Faculty of Medicine, Padjadjaran University, and Dr. Hasan

Sadikin General Hospital. Verina Logito is a clinical pathologist and staff member of the Immunoserology Department, Faculty of Medicine, Padjadjaran University and Dr. Hasan Sadikin General Hospital. The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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