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Comparative Study of Cycle Threshold RT-PCR SARS Cov-2 between Saliva Specimen and Nasopharyngeal Swab

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ABSTRACT

Coronavirus Disease 2019 (Covid-19) is an infectious disease that spreads quickly and attacks the respiratory system that can causing death. The main diagnosis of Covid-19 is conducted by a nasopharyngeal swab, an invasive method which can in turn increase the risk of transmission from patient to swabber, and cause discomfort for the patient when nasopharyngeal swab was collected. Hence, there is a need for non-invasive methods development, one of which is using saliva specimens. This study aims to evaluate the potential of using saliva specimens for diagnosis as an alternative to nasopharyngeal swabs. The study was conducted on confirmed patients at Hajj Dormitory Embarkation Surabaya using an analytical experimental research design. The samples were collected by simple random sampling from 35 patients at Hajj Dormitory Embarkation Surabaya who meet the inclusion criteria, and evaluated at Surabaya Regional Health Laboratory using RT-PCR (Real Time Polymerase Chain Extraction). The results showed that there was no significant difference between the cycle threshold RT-PCR of nasopharyngeal swab and saliva specimen, for target E Gene, OrF1ab Gene, and N Gene. The sensitivity and specificity of saliva specimens are 88.2% and 100%, respectively, from nasopharyngeal swabs. Hence, saliva specimen has the potential to be used as a non-invasive method for Covid-19 diagnosis and for patient comfort.
Keywords: Covid-19; Saliva specimen; Nasopharyngeal swab; Target genes

INTRODUCTION

Coronavirus Disease 2019 (COVID-19) is a recent infectious disease that attacks the respiratory system with an incubation period of 5-14 days with general symptoms similar to symptoms of acute respiratory disorders such as fever, cough, and shortness of breath, but severe symptoms can cause pneumonia, acute respiratory syndrome, kidney failure, and even death[1].

The Covid-19 case was first reported in Wuhan, China in December 2019, where 44 people were suffering from mysterious pneumonia. Research showed that the case was caused by a new type of beta coronavirus, which was later named the 2019 novel Coronavirus (2019-nCoV). To date, there have been 13,540,548 new cases of Covid-19 in the world[2].

Transmission of Covid-19 which is quite high has an impact on the need for fast, accurate, and convenient diagnostic tests that can immediately identify, isolate, and treat patients to reduce the mortality rate and the risk of spreading infection in the community. There are various stages of examination to diagnose Covid-19, starting with a clinical history of the patient and travel history, supporting examinations such as chest X-rays or antigen-antibody tests, and PCR examinations[3].

The standard examination for the diagnosis of SARS-CoV-2 from WHO is based on the detection of genetic material through nucleic acid amplification tests, such as real-time reverse-transcription polymerase chain reaction (rRT-PCR) with target genes E, RdRP, N, and S[2]. Another examination is the Rapid Diagnostic Test (RDT) for antibodies and/or antigens to screen for coronavirus infection in a short time[3].

Specimens used in carrying out the Covid-19 diagnostic test can be taken from the upper respiratory tract (nasopharyngeal swab, oropharyngeal swab), lower respiratory tract (endotracheal aspiration sputum, or bronchoalveolar lavage) in patients with severe respiratory disease, specimens from blood and faeces[4].

Taking a nasopharyngeal swab for a Covid-19 diagnostic examination is an invasive method. The discomfort felt by the patient and the presence of several contraindications can be an obstacle to taking

specimens from the nasopharyngeal swab. This method requires trained medical personnel and requires the use of personal protective equipment because this activity has the potential to infect medical personnel when the specimen is collected[5].

One of the specimens that can be taken by a non-invasive method is saliva. The saliva secreted by the saliva glands contains water, electrolytes, mucus, as well as digestive proteins, and other organic molecules. Saliva is produced by three pairs of major glands, namely the submandibular, sublingual, and many minor glands scattered around the oral cavity. Although it originates from several glands, saliva is widely used and can be used as a diagnostic specimen to identify the existence of pathogens in the oral cavity[6]. Taking saliva specimens does not require specific sample handling. Hence, saliva is a promising specimen for diagnosis. Approximately 69% of studies with saliva specimens showed a sensitivity that was not much different from nasopharyngeal swab[7]. Another study revealed that the saliva specimen specificity was 98.9% of the PCR examination.

This study was conducted to evaluate the potential of using saliva specimens for diagnosis instead of nasopharyngeal swabs from confirmed patients at Hajj Dormitory Embarkation Surabaya.

METHODS

The research was of analytical experimental design. Specimen collection was carried out at Hajj Dormitory Embarkation Surabaya, which is the centralized isolation location for confirmed Covid-19 patients in May 2022, while specimen examination using the RT PCR test was carried out at the Biosafety Level 2 (BSL-2) Surabaya Regional Health Laboratory that is one of the Covid-19 referral laboratories in Surabaya. The research was conducted from May–August 2022. The samples were collected using simple random sampling with the inclusion criteria as follows: patients who come one day after confirmation of Covid-19 in the centralized isolation at the Hajj Dormitory Embarkation Surabaya and are willing to take part in the study by filling out informed consent by the patient or by the patient's family (if the patient cannot communicate). If the patients stayed more than one day in centralized isolation at the Hajj Embarkation Dormitory Surabaya but refused to participate in the study, they were categorized as exclusion criteria and were not selected as samples for the research. Based on the sample calculation, the sample size in this study is 35 respondents. Before specimens were collected, the patients had to sign informed consent after reading the ethical clearance No. KE/V/2022 University of Surabaya.

Saliva specimen collection

A sampling of saliva is carried out by asking the patient to rinse his mouth using mouthwash from the Gargle Solution tube for 10-15 seconds while tilting his head back (make sure the gargling hits the throat). Then stop for a while holding the mouthwash in your mouth, then repeat the gargling motion 3 times. When finished, the mouthwash is removed from the mouth into the Gargle Solution tube using a funnel (adapter). The tube containing the mouthwash is then added to the Mixing Solution (Collection Buffer) and shaken until frothy. The tube is labeled and stored in a box at room temperature and sent to the examining laboratory. The patients must fill out and sign the informed consent form. The data used were primary data from respondents and interviews. In the study, the data obtained were analyzed statistically and presented in the form of a frequency distribution table. This statistical analysis was carried out by SPSS statistics using Fisher's test and the ANOVA test.

Nasopharyngeal swab collection

Sampling from the nasopharynx is carried out by inserting a cotton swab through the nostril parallel to the palate until an obstacle is felt or the distance is equivalent to the distance from the patient's ear to the nostril, which indicates contact with the nasopharynx. The wipe should be lightly rubbed and gently rolled. Leave the swab in

place for a few seconds to absorb secretions. Slowly remove the swab by rotating it. Then the swab cotton is put into the VTM (Viral Transport Medium) bottle and packaged and labeled in a standard way and then stored in a cool box filled with cool pack and sent to the examining laboratory.

RNA Extraction

RNA extraction from the sample was conducted in Level 3 Personal Protective Equipment using the Mag-Bind RNA Extraction Kit Maccura RNA/DNA reagent with an AllSheng brand machine. Sample amplification was conducted using Sansure reagent with Tianlong 96 brand RT-PCR machine.

Cycle Threshold (CT)

After the RT-PCR process was completed, data analysis was carried out based on the instructions from the manual reagent. The analysis must be carried out separately for each target gene using a manual threshold line setting. The threshold line must be adjusted to be within the exponential phase range of the fluorescence curve as well as above the background signal. The NC (Negative Control) result must be negative and not show an amplification curve. If the NC shows an amplification curve, it is an indication of contamination. Immediately repeat the preparation of the master mix reagent by paying attention to the accuracy and safety of reagent quality control. PC (Positive Control) results show an amplification curve with a range of CT values between 30-35. If the PC shows an amplification curve of CT > 35 or negative, immediately repeat the master mix reagent preparation by paying attention to the accuracy and safety of reagent quality control. The IC (Internal Control) results show an amplification curve with a CT < 35, indicating the presence of RNA genetic material from human specimens. The IC value indicates that the nucleic acid process and specimen collection have been carried out correctly.

RESULTS AND DISCUSSION

Results

The research results were obtained through primary and secondary data collection that was carried out in May 2022 at Hajj Dormitory Embarkation Surabaya, which is a centralized central isolation location for confirmed Covid-19 patients. This study used saliva specimens and nasopharyngeal swabs taken from 35 confirmed Covid-19 patients who were treated for at least one day at Hajj Dormitory Embarkation Surabaya. The collected specimens were then examined using the RT-PCR (Real-Time Polymerase Chain Reaction) method at the Surabaya Regional Health Laboratory which is the Covid-19 reference laboratory for the Surabaya City area.

Characteristics of research respondents (Table I) based on gender, age, and symptoms felt during the research. Most of the respondents were female with a percentage of 62.9% and most of the respondents were aged in the range of 21-30 years with a percentage of 25.7%. The results of the study also showed that the respondents who took part in the study were in various age ranges starting from children with a percentage of 2.9% and the elderly with a percentage of 17.1%.

Table II below shows that most of the RT PCR tests from saliva specimens and nasopharyngeal swabs showed positive results for Covid-19. In positive results, the number of positives was found more for nasopharyngeal swabs, namely 97.1% than for saliva specimens, 85.7%. Whereas negative results were found more frequent for saliva specimens, 14.3% than for nasopharyngeal swabs, 2.9%. Statistical tests showed that the p value was 0.656, which means there was no significant difference in results between RT-PCR examination of saliva specimen and nasopharyngeal swab in this study.

Table I. Characteristics of Respondents

	Characteristics	*Number of sample*	*%*
Gender	Male	13	37.1
	Female	22	62.9
Age	1 – 10 years	1	2.9
	11 – 20 years	4	11.4
	21 – 30 years	9	25.7
	31 – 40 years	7	20.0
	41 – 50 years	3	8.6
	51 – 60 years	5	14.3
	> 60 years	6	17.1
	ARI (Acute Respiratory Infections)	31	88.6
	Non ARI	7	20.0
	Asymptomatic	2	5.7

Table II. Frequency of positive and negative result for saliva specimen and nasopharyngeal swab in RT-PCR results out of 35 total samples

Result	*Saliva specimen*		*Nasopharyngeal swab*		*P value*
	n	*%*	*n*	*%*	
Positive	30	85.7	34	97.1	
Negative	5	14.3	1	2.9	0.656
Total samples	35	100.0	35	100.0	

n = number of samples

Table III. Conformity of Saliva Specimen to Nasopharyngeal Swab in RT-PCR Result

Result	*Positive results for nasopharyngeal swab*		*Negative results for nasopharyngeal swab*		*Total samples*		*P value*
	n	*%*	*n*	*%*	*n*	*%*	
Positive for saliva specimen	30	85.7	0	0	30	85.7	
Negative for saliva specimen	4	11.4	1	2.9	5	14.3	0.143
Total samples	34	97.1	1	2.9	35	100.0	

n = number of samples

Based on the results in Table III, the sensitivity value is calculated using the formula [a/(a+c)x100%] so that [30 / (30+4) x 100% = 88.2%] is obtained which indicates that the sensitivity value of the saliva specimen as a diagnostic tool to confirm Covid-19 at 88.2%. Meanwhile, the specificity will be calculated using the formula [d/(b+d) x100%] to obtain the calculation [1/(0+1) x 100% = 100.0], which shows the specificity of the saliva specimen as a diagnostic tool to confirm Covid-19 19 by 100%. The statistical test showed a p-value of

Table IV. Conformitybetweentarget genedetection for saliva specimen and nasopharyngeal swab in RT-PCR examination

Target gene	*Result*	*Detected in nasopharyngealswab*		*Undetected in nasopharyngealswab*		*Total*		*P value*
		n	*%*	*n*	*%*	*n*	*%*	
Gene E	detected in saliva specimen	35	100.0	0	0.0	35	100.0	-
	undetected in saliva specimen	0	0.0	0	0.0	0	0.0	
Gene OrF1ab	detected in saliva specimen	29	82.9	0	0.0	29	82.9	0.17
	undetected in saliva specimen	5	14.3	1	2.9	6	17.1	
Gene N	detected in saliva specimen	32	91.4	0	0.0	32	91.4	-
	undetected in saliva specimen	3	8.6	0	0.0	3	8.6	

n = number of samples

0.143 which means that there was no significant relationship between the results of saliva specimen RT-PCR examination compared to examination of nasopharyngeal swab.

Based on the results in Table IV, the sensitivity value of gene E is calculated using the formula [a/(a+c)x100%] so that [35 / (35+0) x 100% = 100.0%] is obtained which indicates that the sensitivity value of gene E is E in saliva specimen as a diagnostic tool to confirm Covid-19 is 100.0%. Meanwhile, the specificity value of gene E cannot be calculated because there is no undetected E gene. The p-value also cannot be tested statistically because there are values that contain 0 because there is no undetectable gene E.

The sensitivity value of the OrF1ab gene is calculated using the formula [a/(a+c)x100%] so that [29 / (29+5) x 100% = 85.29%] is obtained which indicates that the sensitivity value of the gene is OrF1ab saliva specimen as a diagnostic tool to confirm Covid-19 by 85.29%. While the specificity will be calculated by the formula [d/(b+d) x100%] so that the calculation [1 / (0+1) x 100% = 100.0] is obtained, which shows the specificity of the OrF1ab gene in saliva specimen as a diagnostic tool to confirm Covid-19 is 100%. The statistical test showed a p-value of 0.171, which means that there was no significant difference between the results of the saliva specimen and nasopharyngeal swab for the OrF1ab RT-PCR gene examination.

The sensitivity value of gene N is calculated using the formula [a/(a+c)x100%] so that [32 / (32+3) x 100% = 91.43%] is obtained which indicates that the sensitivity value of gene N in saliva specimen as a diagnostic tool to confirm Covid-19 is 91.43%. Meanwhile, the specificity value of gene N cannot be calculated because there is no undetected N gene. The p-value also cannot be tested

statistically because there are values that contain 0 because there is no undetectable gene N.

Discussion

In this study, it was found that the number of positive results from saliva specimens was almost the same as the number of positive results from nasopharyngeal swabs. This is in line with research that showed that out of 153 nasopharyngeal swabs, 119 of them had positive results and the remaining 34 specimens had negative results. As for saliva, of the 153 tested, 105 specimens were positive and 48 were negative[5]. Research conducted by Wang To at a health facility in Hong Kong also showed that the Covid-19 virus was detected in 11 of the 12 Saliva specimens examined[8]. This could indicate that the false positive values obtained from saliva specimens are quite small so the sensitivity of saliva as a diagnostic tool of choice to replace nasopharyngeal swabs for the detection of Covid-19 can be considered.

Statistical test results showed that there was no significant relationship between RT PCR examination using saliva specimen and nasopharyngeal swab with p=0.656. In diagnosing for sure COVID-19, the RT-PCR method is the most standard because it is sensitive, specific, and capable of processing large numbers of samples. The gap between sample size and capacity to perform RT-PCR promptly is considered a major limitation of the public health containment strategy. Therefore, there is a need for alternative tests, especially RDT, which are time-efficient, easy to perform, and can be used for point-of-care (POCT) or community-based testing. This RDT antigen-based immunofluorescence assay shows high sensitivity and specificity in respiratory samples and is obtained from patients who mainly come during the first week of being infected COVID-19[9].

Covid-19 examination uses the RT-PCR (Reverse Transcription Polymerase Chain Reaction) method, which so far is the main method, used as a determinant of the diagnosis of Covid-19. Clinical specimens for RT-PCR can be obtained from the upper respiratory tract via oropharyngeal swabs or swabs or from Broncho Alveolar Lavage (BAL), or tracheal aspiration. The target gene that determines CT (Cycle Threshold) varies in each examination, based on the reagent used during the PCR examination. In this research, the target genes used were the Envelop Protein gene (E), the OrF1ab gene, and the Nucleocapsid gene (N). The statistical test results in this study showed that the sensitivity value of the saliva specimen in determining the diagnosis of Covid-19 was 88.2%. Meanwhile, the specificity value of the saliva specimen as a diagnostic tool to confirm Covid-19 is 100%. This is similar to a study conducted in Thailand where 100 patients were asked to take saliva specimens independently. This study showed that the sensitivity value of the saliva specimen was 84.2% and the saliva specimen specificity was 98.9% for the Covid-19 PCR examination[10].

Examination of the RT PCR results for the saliva specimen and nasopharyngeal swab based on the OrF1ab gene test showed a sensitivity value of 85.29% and a specificity value of 100.0%. There was no significant difference between the results for the saliva specimen and nasopharyngeal swab OrF1ab RT-PCR gene examination with p = 0.171. This is inconsistent with a study that stated that the p-value of the OrF1ab gene for saliva specimen and nasopharyngeal swab examination was 0.004. This difference can be caused by the different number of respondents, the technique of collecting specimens, the technique of storing specimens, the PCR reagents used, and the method of reading the results[11].

Examination of the RT PCR results for the saliva specimen and nasopharyngeal swab based on gene N showed a sensitivity value of 91.43% but the specificity value could not be detected because no N gene appeared because there were no negative results in the statistical test, so the p-value also could not be tested statistically. This is inconsistent with a study conducted by Yusuf et al (2022) which stated that the p-value of gene N on examination of

the saliva specimen and nasopharyngeal swab was 0.004. This difference can be caused by the different number of respondents, the technique of collecting specimens, the technique of storing specimens, the PCR reagents used, and the method of reading the results[11].

In a study conducted at Ramathibodi Hospital in Thailand, 2 patients were found to be positive for Covid-19 through saliva specimen but were confirmed negative through PCR examination of the nasopharyngeal swab. The two samples each had an OrF1ab CT value of 33.9 and 34.8 and a gene N CT value of 36.2 and 33.7 respectively, where both gene values can be said to be positive for Covid-19. The two patients also experienced anosmia, which is one of the symptoms of Covid-19. Therefore, PCR examination using saliva specimens can be used as a complementary diagnostic test for Covid-19 examination[10].

Research related to saliva specimens was also conducted in Kuwait on 891 respondents where two specimens were also taken for each respondent, namely saliva specimen and nasopharyngeal swabs. The results of this study showed that out of 891 respondents, 344 respondents (38.61%) were confirmed positive for Covid-19 with nasopharyngeal swabs, and 287 respondents (83.43%) of them were also confirmed positive based on examination of saliva specimens. The sensitivity value is 83.43% and the specificity value is 96.71%[12].

The sensitivity and specificity values of the saliva specimen were quite high—although slightly below the sensitivity and specificity values of the nasopharyngeal swab—indicating that the SARS CoV-2 virus was also found in the saliva specimen, which could be detected by PCR examination. The SARS CoV-2 virus, resulting in the production of infected saliva as well, infects epithelial cells lining the ducts of the minor saliva glands, which express ACE2. In addition, the visibility of the virus can be identified in saliva samples in two ways, namely with and without coughing[13].

There are several advantages to using saliva as a Covid-19 detection tool. First, taking saliva specimens is a non-invasive procedure method that can reduce the risk of nosocomial transmission of Covid-19, where the risk of transmission to health workers who carry out swabs can be reduced. Second, the place where the saliva specimen is collected does not require a special room or closed room, as is the case with nasopharyngeal swab collection; this can make it easier to collect saliva specimens that can be done outside the health facility. Third, taking a saliva specimen requires less time than taking a nasopharyngeal swab, and can be done even by non-health workers to take the specimen[8].

CONCLUSION

In this study, CT examinations were carried out for RT PCR tests for SARS CoV-2 from saliva specimens and nasopharyngeal swabs for 35 patients who carried out central isolation at Hajj Dormitory Embarkation Surabaya. The results of the study showed that the number of patients who were confirmed positive for Covid-19 did not differ too much between saliva specimens and nasopharyngeal swabs. The results of RT PCR examination for each gene E, gene OrF1ab, and gene N showed no significant differences in saliva specimens and nasopharyngeal swabs. The sensitivity value for the saliva specimen to the nasopharyngeal swab was 88.2% and the specificity value was 100%. This shows that saliva specimens can be used as an alternative for diagnosis of Covid-19, plus other advantages such as taking saliva specimens is a non-invasive method, can be carried out outside the special swab room, and taken by non-health workers.

CONFLICT OF INTEREST

The authors have no conflict of interest that might be construed to influence the results or interpretation of this manuscript.

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