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The Impact of Cycle Threshold Value in Influencing the Performance of COVID-19 Antigen

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ABSTRACT

COVID-19 antigen is an alternative test for detecting SARS-CoV-2 infection. Viral load represented by the Cycle Threshold (CT) in the Real-Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) affects the diagnostic performance of the test. Higher CT values result in reduced sensitivity of the SARS-CoV-2 antigen. The main objective of this study was to determine the highest CT value in rRT-PCR that still yielded reactive results in the COVID-19 antigen test. This cross-sectional study was conducted at the Fever Outpatient Clinic in Dr. Cipto Mangunkusumo Hospital from July 2020 to June 2021. Two hundred and thirty-five naso-oropharyngeal swabs were taken from patients with confirmed and suspected COVID-19 diagnoses. About 24.7% of subjects were tested positive. The median highest CT value giving reactive COVID-19 antigen results was 28.22 (13.33-39.16), while the median CT value for non-reactive antigen results was 34.45 (26.08-39.65). At a CT value ≤ 40, the COVID-19 antigen test demonstrated 63.8% sensitivity, 99.4% specificity, 89.3% Negative Predictive Value (NPV), and 97.4% Positive Predictive Value (PPV). At the CT value ≤ 25, the test showed 92.3% sensitivity, 99.4% specificity, 99.4% NPV, 92.3% PPV, 163.4 LR+, and 0.1 LR-. The identified cut-off point for the CT value was 29.82, with a sensitivity of 64.9% and specificity of 81%. In conclusion, COVID-19 antigen is a valuable test for screening patients with symptoms of SARS-CoV-2 infection. Understanding the influence of cycle threshold can enhance the interpretation and reliability of the antigen test.

Keywords: Cycle threshold, COVID-19 antigen

INTRODUCTION

Coronavirus Disease 2019 (COVID-19) is a severe acute respiratory distress syndrome caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infections.[1] After being declared a pandemic by the World Health Organization (WHO) in early 2020 until April 17, 2021, COVID-19 has spread to 223 countries, infected 139,501,934 people, and killed 2,992,193 people.[1] The number of cases continues to grow, especially in developing and developed countries. Indonesia is one of the countries in Southeast Asia with the highest number of cases and deaths.[1] On April 14, 2021, it was reported that 1,583,182 people had been infected with COVID-19, and 42,906 (2.7%) died.[1] This caused a substantial economic and health burden.

Real-Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) is one of the nucleic amplification tests used to detect SARS-CoV-2 infection.[2] WHO recommends it for screening, diagnosis, and evaluating therapy in patients who are suspected of SARS-CoV-2 infection.[2] rRT-PCR results are reported as an amplification curve and Cycle Threshold (CT). CT is the number of rRT-PCR replication cycles in which SARS-CoV-2 RNA begins to be detected.[2] A lower CT result indicates a higher amount of virus; therefore, CT is indirectly related to the SARS-CoV-2 viral load.[2]

The increase in COVID-19 cases caused the overcapacity of the rRT-PCR test, with the number of orders being higher than the turnaround time (TAT) required to process samples.[3] The SARS-CoV-2 antigen test was invented to reduce the test burden. A systematic review by Cochrane showed that the SARS-CoV-2 antigen had a sensitivity of 56.2% (95% CI 29.5%-79.8%) and specificity of 99.5% (95% CI 98.1%-99.9%).[3]

The CT on the rRT-PCR test is one factor influencing the SARS-CoV-2 antigen. A high viral load will give a low CT value. Marca *et al.* found that the NPV of the SARS-CoV-2 antigen was 97% at CT ≤30 but was reduced to 32% at CT ≤40.[4] Cochrane systematic review also showed that the sensitivity of the SARS-CoV-2 antigen was reduced, especially in samples with low viral loads. A sensitivity of 93.2%

(95% CI 63.6%-99.1%) and decreased sensitivity of 32.6% (95% CI 17.5%-52.6%) were found in specimens with high and low viral load, respectively.[3] This indicates that the SARS-CoV-2 antigen is only helpful for patients with high SARS-CoV-2 viral loads. The research was driven by the absence of data from Indonesia and differences in populations, methodologies, and the utilization of various reagent kits that might have influenced disparate outcomes. The study aimed to determine the highest CT value in rRT-PCR that yielded reactive results in the COVID-19 Antigen test. Secondary objectives were to assess sensitivity, specificity, Negative Predictive Value (NPV), Positive Predictive Value (PPV), Likelihood Ratio Positive (LR+), and Likelihood Ratio Negative (LR-) of COVID-19 antigen at a predetermined CT value cut-offs and measure the CT value cut-off point, which gives the optimum sensitivity and specificity.

METHODS

This study was an observational research using a cross-sectional method performed at Dr. Cipto Mangunkusumo Hospital, Jakarta, from July 2020 to June 2021. The research subjects were all patients suspected and confirmed with SARS-CoV-2 infections at Dr. Cipto Mangunkusumo Hospital Fever Outpatient Clinic. Inclusion criteria were patients suspected and confirmed with COVID-19 who were verified by the Indonesian Ministry of Health operational definition and agreed to participate in the study by signing the informed consent form.[5] Patients without complete medical records and the Indonesian Ministry of Health epidemiological form were excluded from this study.

The specimen was collected from a total of 235 subjects who were included in the study. Trained physicians collected nasopharyngeal and oropharyngeal specimens. A flocked swab was passed through the patient's nostril to the posterior nasopharynx, left for several seconds, and was then removed while rotating and reinserted into the mouth's posterior palate, roof, and peritonsillar pillar. The swab was placed in a sterile viral transport medium, sealed, labeled, and sent for rRT-PCR within 2-8^{0}C. rRT-PCR was performed immediately for most of the specimens using Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic Kit (PCR-Fluorescence Probing)®Sansure Biotech Inc for SARS-CoV-2 ORF1ab and N target gene. The rest of the specimens were tested using Real-Q 2019-nCoV Detection Kit®Biosewoon Inc for RdRp and E target gene. The assay was conducted according to the manufacturer kit by a trained medical technician. CT values were determined based on the highest CT values reported by a target gene (ORF1ab, N, RdRp) for positive rRT-PCR test producing two CT values. E target gene from Real-Q 2019-nCoV Detection Kit® Biosewoon was not specific for SARS-CoV-2 and hence was not included in the analysis.

COVID-19 antigen test was performed on all patients before specimen collection for rRT-PCR by trained physicians. A flocked swab was gently inserted through the nostril to a depth of 5 to 7 cm toward the posterior nasopharynx, left for several seconds, and was then slowly removed while rotating. Specimens were inserted into a sterile plain vacutainer tube and stored in an incubator at 2-8^{0}C. Specimens were collected within 4 hours and analyzed using the standard Q COVID-19 Ag® SD Biosensor under a biosafety cabinet 2A. A trained medical technician performed the assay and interpreted it according to the manufacturer's kit. Two bands on the control and test band indicated positive antigen test results. No reaction on the control band indicated an invalid result, whereas a negative result was only indicated by a line on the control band.

Data of age, gender, comorbidity, disease onset, time interval between symptoms and rRT-PCR test, contact history, symptoms, severity, result of rRT-PCT (including CT values), and the result of COVID-19 antigen test. Data were processed using SPSS 20 and MeldCalc software. Kolmogorov-Smirnov test was used to determine the normality of numeric variables. Median, minimum, and maximum CT values needed to give reactive results on the COVID-19 Ag test were calculated and analyzed with the Mann-Whitney U test. The diagnostic performance of the COVID-19 antigen, including its sensitivity, specificity, PPV, NPV, LR+, LR-, prevalence, and accuracy, was determined using table 2x2 against results from the rRT-PCR as the gold standard at different predetermined CT values cut-off of CT ≤ 25, CT ≤ 33 and CT ≤ 40.[3] CT value cut-off point, which gives the optimum sensitivity and specificity was measured using Youden Index derived from ROC curve and Area Under Curve (AUC). A 95% confidence interval was reported along with the calculation result. A p-value ≤ 0.05 was considered statistically significant.

Research permission was obtained with ethical approval from the Faculty of Medicine Ethics Committee, University of Indonesia, with article number 595/UN2.F1/ETIK/PPM.00.02/2020.

RESULTS AND DISCUSSIONS

There were 235 subjects involved in the study. Among the observed subjects, 24.7% were positive for COVID-19. Characteristics of the research subject

are displayed in Table 1. It was observed in this study that COVID-19 primarily affected adult females (n=42, 18%), particularly those in the younger age group (median age 34.5 years, 17-76 years), with the majority of cases being asymptomatic or exhibiting mild symptoms. Costeria *et al*. demonstrated that this relationship is related to the protective effect of estrogen in modulating the immune response to severe and critical symptoms in females. Estrogen modulates the response of B cells through T helper 2 to produce higher levels of antibodies while also reducing the T helper 1 response in cellular immunity. Therefore, estrogen is considered able to protect the body from severe and critical symptoms of COVID-19.[6] On the other hand, subjects with negative rRT-PCR results (n=177, 75.3%) mainly were younger

Table 1. Characteristics of research subjects

Variable (n=235)	Positive rRT-PCR	Negative rRT-PCR
rRT-PCR, n(%)	58 (24.7)	177 (75.3)
Gender		
Male, n(%)	16 (6.9)	96 (41.2)
Female, n(%)	42 (18)	79 (33.9)
Age (years)	34.5 (17-76)	17 (0-49)
Disease onset (days)	2 (0-24)	7 (0-30)
The time interval between specimen collection and rRT-PCR test result (days)	1 (0-3)	0 (0-2)
Contact history		
Present, n(%)	53 (23.6)	153 (68)
Absent, n(%)	4 (1.8)	15 (6.7)
Symptoms, n(%)	45 (20.3)	118 (53.2)
Fever, n(%)	13 (5.9)	58 (26.2)
Temperature (^{o}C)	38 (36.6-39)	38.3 (36.9-40.8)
Cough, n(%)	29 (13.1)	79 (35.6)
Congested nose, n(%)	21 (9.5)	61 (27.5)
Sore throat, n(%)	12 (5.4)	8 (3.6)
Dyspnea, n(%)	8 (3.6)	6 (2.7)
Shivering, n(%)	2 (0.9)	3 (1.4)
Headache, n(%)	10 (4.5)	3 (1.4)
Malaise, n(%)	14 (6.3)	10 (4.5)
Myalgia, n(%)	5 (2.3)	2 (0.9)
Nausea and/or vomiting, n(%)	4 (1.8)	7 (3.2)
Abdominal pain, n(%)	1 (0.5)	2 (0.9)
Diarrhea, n(%)	2 (0.9)	11 (5)
Anosmia, n(%)	17 (7.7)	3 (1.3)
Others, n(%)	2 (0.9)	1 (0.5)
Severity		
Asymptomatic, n(%)	11 (5)	-
Mild, n(%)	37 (16.7)	-
Moderate, n(%)	5 (2.3)	-
Severe, n(%)	1 (0.5)	-
Critical, n(%)	2 (0.9)	-
Comorbidity n(%)	20 (9)	65 (29.3)
Pregnancy, n(%)	1 (0.5)	1 (0.5)
Diabetes, n(%)	6 (2.7)	1 (0.5)
Heart disease, n(%)	1 (0.5)	10 (4.5)
Hypertension, n(%)	11 (5)	4 (1.8)
Malignancy, n(%)	4 (1.8)	8 (8.1)
Immunological disease, n(%)	0 (0)	11 (5)
Chronic kidney disease, n(%)	2 (0.9)	5 (2.3)
Chronic liver disease, n(%)	0 (0)	3 (1.4)
Others, n(%)	5 (2.3)	22 (9.9)

(median age of 17 years old, 0-49 years). On the other hand, pediatric patients were subjects that have been widely investigated in research (n=149, 63.4%), but COVID-19 was only confirmed in a small portion of the population (n=10, 0.04%). Children who were confirmed positive for COVID-19 also showed mild symptoms or were asymptomatic. This result might be caused by lower expression of ACE2 receptors in children, undeveloped immune responses, and competition with other infections leading to milder symptoms.[7] In addition, the recommendation from the Indonesian Pediatrician Association regarding online education during the COVID-19 pandemic was also presumed to be another reason.[8] The most common symptoms in patients with positive rRT-PCR were persistent cough, congested nose, anosmia, weakness, fever, sore throat, headache, dyspnea, myalgia, and nausea or vomiting. This result was similar to the published data by Indonesian Health Ministry.[9]

The median CT values in this study's reactive COVID-19 antigen test were lower than the non-reactive COVID-19 antigen test (Figure 1). Among reactive COVID-19 antigen tests (n=37), the median CT value was 28.22 (13.33-39.16), where as for non-reactive tests (n=21), the median CT value was 34.45 (26.08-39.65). Statistical analysis yielded a p-value of less than 0.01, signifying a significant difference between the groups. This finding suggests a substantial viral load or concentration disparity between reactive and non-reactive tests, as indicated by their CT values. The result was consistent with Cerutti *et al*., which reported median CT of 22.3 and 32.1, respectively. This discrepancy might be due to differences in the study population, which included patients with moderate, severe, and critical symptoms. Different rRT-PCR reagents and the amplified genes might also cause the difference.[10]

This study showed that the performance of the COVID-19 antigen test was good. This study had good accuracy (90.6%), sufficient sensitivity (63.8%), high specificity (99.4%), high LR+ (112.9), low LR- (0.4), high PPV (97.4%), and high NPV (89.3%)

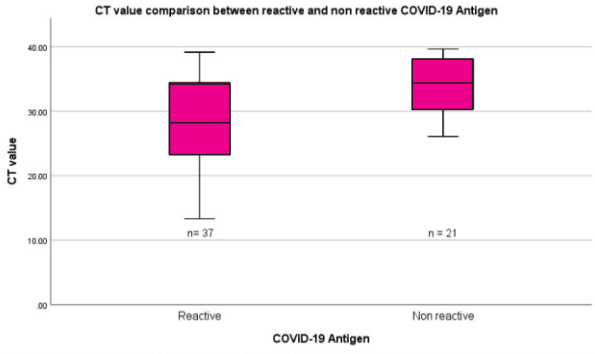

Figure 1. Comparison of CT values between reactive and non-reactive COVID-19 antigen test results

(Table 2). There were differences in the results between this study and Dinnes *et al*., which reported a lower sensitivity of 56.2 (29.5-79.8%) and a similar specificity of 99.5% (98.1-99.9%). The difference might be caused by several factors, such as limited studies that were collected at the start of the 2020 pandemic, which might include some antigen tests that were not commercially available due to low sensitivity issues and differences in the population studied. Dimas *et al.* analyzed three of the five studies, which consisted only of suspected COVID-19 population, in contrast to this study, which examined both suspected and COVID-19-confirmed patients. The systematic reviews also included all research carried out in developed countries and excluded data from developing countries. These differences might cause differences in sensitivity.[3]

One of the factors affecting the performance of the COVID-19 antigen test is CT values. There was an increase in sensitivity, LR+, NPV, and accuracy, and a decrease in LR at CT < 25 and < 33. Dinnes *et al.* also supported the idea that there was a change in sensitivity in low and high CT. The sensitivity of the COVID-19 antigen test at CT 25 and CT >25 was 94.5% and 40.7% (31.8-50.3%), respectively.[3]

This study also measured the CT value cut-off point, which gives the optimum sensitivity and specificity. According to the ROC curve, AUC of 0.775

Table 2. Diagnostic performance of COVID-19 antigen test at different predetermined CT values

Variable	CT < 25	CT< 33	CT < 40
Sensitivity (95% CI)	92.3 (63.9-99.8)	77.1 (59.9-89.6)	63.8 (50.1-76.1)
Specificity (95% CI)	99.4 (96.9-99.9)	99.4 (96.9-99.9)	99.4 (96.9-99.9)
PPV (95% CI)	92.3 (62.8-98.8)	96.4 (79.1-99.5)	97.4 (83.9-99.6)
NPV (95% CI)	99.4 (96.4-99.9)	95.7 (92.3-98.1)	89.3 (85.6-92.2)
LR +(95% CI)	163.4 (23-116.8)	136.5 (19.2-972.1)	112.9 (15.8-804.8)
LR- (95% CI)	0.1 (0.01-0.5)	0.2 (0.1-0.4)	0.4 (0.3-0.5)
Prevalence (95% CI)	6.8 (3.7-11.4)	16.5 (11.8-22.2)	24.7 (19.3-30.7)
Accuracy (95% CI)	98.9 (96.3-99.9)	95.8 (92.1-98.1)	90.6 (86.2-94.1)

Abbreviation: CL, Confidence Interval; PPV, Positive Predictive Value; NPV, Negative Predictive Value; LR, Likelihood Ratio

(95% CI: 0.656-0.893), the cut-off point for the CT value was 29.82 with a sensitivity of 64.9% and specificity of 81%, p<0.01. Several studies determine the CT cut-off point, which provides the best sensitivity and specificity. Nalumansi *et al*. and Bruzzone *et al*. found that the CT limit was 29.[11,12] The population of the studies influenced the difference in cut-off points in other studies.

The results of this study demonstrated that the COVID-19 antigen testwas below the qualifications expected by WHO (sensitivity of ≥ 80%). Therefore, negative results could not exclude SARS-CoV-2 infection. However, the test's specificity was high and exceeded the criteria set by WHO (specificity ≥ 97%). Based on this, the test could be used for diagnosis. The reactive results of COVID-19 Ag on contact tracing or community surveillance can be used to confirm SARS-CoV-2 infection, whereas non-reactive result requires confirmation through rRT-PCR testing. Using COVID-19 antigens requires further consideration regarding the ability of rRT-PCR in an area, ease of use, and TAT. Easy-to-use tests are needed in urgent conditions; the rRT-PCR test is limited to spikes in cases, whereas the COVID-19 Ag test is an alternative test.

This research is one of the first in Indonesia to evaluate COVID-19 antigen testing, highlighting the critical role of CT values in influencing test sensitivity and specificity. This knowledge can improve clinical decisions, optimize resource allocation, inform public health strategies, and guide future test development, ultimately enhancing the management of COVID-19 in Indonesia. It can also be applied to other diseases, which has a similar principle to the antigen test used in this study. The population studied included subjects with suspected and confirmed COVID-19 in the hospital outpatient unit with mild symptoms and asymptomatic. Therefore, this research was one of the studies that represent a realistic situation in which the test is commonly used.

One limitation of the study was the use of a nontraditional approach to perform COVID-19 antigen testing. Specifically, the sample was collected in a well-ventilated area and subsequently transported to the laboratory, where further analysis was conducted in a biosafety cabinet. This was not a common procedure, although the previous unpublished validation test was carried out, and the method was considered valid.

CONCLUSIONS AND SUGGESTIONS

The highest median CT value in the reactive COVID-19 antigen test was 28.22. Sensitivity, specificity, NPV, PPV, LR+, and LR- of COVID-19 antigen were highest at CT < 25 compared to CT < 33 and CT < 40. The optimum sensitivity and specificity were obtained at the CT value cut-off point of 29.82.

While the COVID-19 antigen's sensitivity was below WHO criteria standards, the test's specificity was high and exceeded WHO criteria. The test could be used for diagnosis, contact tracing, or community surveillance. The impact of CT values on performance must also be considered for the test's use.

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