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The Validity of Cross Priming Amplification to Detect SARS-CoV-2 Virus

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ABSTRACT

The standard molecular technique to detect the SARS-CoV-2 virus is The Real-Time Reverse-Transcription Polymerase Chain Reaction (rRT-PCR). It requires sophisticated equipment and a time-consuming sample process. The Cross Priming Amplification (CPA) is a nucleic acid amplification technique that amplifies DNA with high specificity and efficiency under constant thermal conditions. This technique is faster than rRT-PCR and doesn't require a biosafety level-2 (BSL-2) facility. The study aimed to determine the validity of CPA with rRT-PCR as a gold standard and to evaluate its performance as molecular rapid tests for detecting SARS-CoV-2 RNA from nasopharyngeal swab specimens. This study was a descriptive diagnostic test by using data retrospectively from swab nasopharyngeal patient samples who were treated at Palabuhan Ratu Hospital with COVID-19 from 01 January to 31 December 2021. The CPA was performed on a total of 52 nasopharyngeal samples at Pelabuhan Ratu Laboratory and rRT-PCR at Provincial Health Laboratory. The validity and correlation tests were performed. The majority of subjects were female between the ages of 34-50 years. The cut-off Tt-value is 3.25, 0.84 Area Under Curve (AUC), with a p-value <0.001. The CPA has good validity for COVID-19 diagnosis with 77% sensitivity, 94% specificity, 96% PPV, and 71% NPV. The sensitivity was increasing with Ct-value <30 (82%) and Ct-value <25 (87%). The CPA had a good validity for the COVID-19 diagnostic test. The CPA could be used as a rapid molecular test for detecting SARS-CoV-2 viral RNA from nasopharyngeal swab specimens.

Keywords: CPA, rRT-PCR, validity

INTRODUCTION

On 30 January 2020, the World Health Organization (WHO) declared a global public health emergency over the outbreak of the new Coronavirus, which was called the 2019 novel Coronavirus (2019-nCoV).[1] On 11 February, WHO officially named the disease Coronavirus Disease 2019 (COVID-19).[2] On 12 February 2021 there have been 107,252,265 confirmed cases of COVID-19 including 2,355,339 deaths reported worldwide. In addition, a total of 1,191,990 cases with 32,381 deaths had been reported in Indonesia.[3]

The SARS-CoV-2 infection manifestation is nonspecific, including respiratory symptoms, fever, cough, dyspnea, and viral pneumonia. The specific diagnostic tests to detect this infection are required to confirm suspected cases, patient screening, and virus surveillance.[4] The rRT-PCR is a standard molecular technique, which is being used to detect COVID-19. This molecular technique has been documented online on the WHO website since 17 January 2020.[5] The rRT-PCR procedure includes Specimen collection; Packing (storage) and shipment of clinical specimens; Communication with the laboratory and information provision; Testing; Reporting. The rRT-PCR technique needs sophisticated laboratory equipment, which usually requires a facility of biosafety level 2 or above. As a consequence, the time required to obtain the results can be up to 2-3 days. In the case of a COVID-19 outbreak, this test is not only extremely time-consuming but is also dangerous since the virus needs to be contained.[5] in order to overcome this problem, it is needed a point of care nucleic acid amplification test (i.e., a rapid, robust, and cost-efficient device that can be used onsite and in the field, which does not necessarily require a trained technician to operate for the detection of COVID-19.[6]

In order to solve time-consuming problems and convoluted laboratory detection techniques using rRT-PCR, Cross Priming Amplification (CPA) can be used as an alternative rapid molecular test. The CPA is an amplification of nucleic acid test (NAAT) technique, which amplifies DNA with high sensitivity, specificity, and efficiency under constant thermal conditions.[7] It is an isothermal amplification reaction

that is carried out by a strand displacement DNA polymerase at a constant temperature of 63^{0}C. The CPA uses multiple cross-linked primers (six to eight primers). This method does not require an initial denaturation step or nicking enzyme. At the temperature of 63^{0}C, thermal activation can cause the spontaneous formation of local denaturation bubbles in the Watson-Crick double strands, thereby increasing the fraction of single-stranded DNA. The formation of primer-template hybrid at transient, spontaneous denaturation bubbles in the DNA template is favor over re-annealing of the template strands by the high concentration of primer relative to DNA template. Strand displacements are encouraged by cross primers with 5'ends, which are not complementary to the template strand and the binding of a displacement primer upstream of the crossing primer. Unlike other isothermal NAAT, the CPA is not defined by only one mechanism but is also defined by the use of one or more cross primers with a 3'end complementary to the target sequence and a non-complementary 5' tail that encodes for an additional sequence. This assay uses Bst DNA polymerase to elongate the primer-template hybrid. The result of CPA is exponential amplification of target DNA with high specificity and sensitivity to produce amplicons.[8]

The advantage of running at an isothermal temperature is reducing the use of thermocycler energy. This technology is more stable and sensitive in the detection SARS-CoV-2 virus compared with rRT-PCR.[7] The CPA can be a candidate for the rapid molecular test in the COVID-19 detection test. Because the CPA does not require sophisticated equipment and is easy to perform, it is an ideal diagnostic instrument for use in remote areas with limited resources.[9] This study aimed to determine the validity of CPA with the rRT-PCR as a gold standard and to evaluate CPA performance as a rapid molecular test to detect SARS-CoV-2 viral RNA from pharyngeal swab specimens.

METHODS

This study was a descriptive diagnostic test by using data collected retrospectively from nasopharyngeal patient swab samples who were treated at Palabuhan Ratu Hospital with COVID-19 from 01 January to 31 December 2021. A total of 52 nasopharyngeal swab samples were collected from patients suspected of COVID-19. The data from swab nasopharyngeal patient swab samples were performed CPA and were then sent to the provincial health laboratory to be tested with rRT-PCR.

The Easy-Nat CPA kit was used for CPA to detect ORF1ab and N gene sequences specific to the novel Coronavirus (2019-nCoV). Through specific amplification primers, specific fluorescent probes, and DNA polymerases with high activity of reverse transcription and chain displacement characteristics, the reaction system can complete the specific amplification process of novel Coronavirus fragments at a single time at a constant temperature, and the fluorescence signal is detected by the applicable instrument and real-time fluorescence curve is automatically generated. The COVID-19-Cartridge is an Internal Standard (IC), which consists of a CPA system that specifically detects human GAPDH mRNA to monitor the effectiveness of sampling, extraction, purification, and amplification reactions. COVID-19-Cartridge was pre-loaded with nucleic acid purification reagents, nucleic acid elution reagents, and CPA reaction reagents. Before the test was started, the COVID-19-RNA extraction solution and the sample were added to cartridge, then the RNA in the sample was purified automatically under the control of the applicable instrument. After the purified RNA and CPA reaction reagent were mixed and heated by the applicable instrument, the constant temperature expansion was carried out, and the fluorescence probe was specifically bound to the target to produce a fluorescence signal. The instrument collected the fluorescence signal in real-time and analyzed the change of the fluorescence signal to determine the test result automatically.[10] Time threshold (Tt) values were calculated for the amplification curves of gene ORF1ab, N, and both Internal Control (IC), respectively, and the results were reported positive or negative by the following criteria: If the Tt-value of gene ORF1ab or gene N is N/A, test result for gene ORF1ab or gene N will be negative. If the Tt-value of gene ORF1ab or gene N < 40, the test result for gene ORF1ab or gene N will be positive; If the Tt-value of an IC is N/A, the test result for the corresponding IC will be negative. If the Tt-value of an IC < 40, the test result for an IC will be positive.

BioRad cfx 96 and Fosun COVID-19 RT-PCR detection kit was used for the detection SARS-CoV-2 RNA. The Fosun COVID-19 RT-PCR detection kit is a fluorescent probe-based TaqMan RT-PCR assay system. Firstly, the RNA of SARS-CoV-2 was reversed transcribed into cDNA by reverse transcriptase, and then PCR amplification was performed with cDNA as a template. During amplification of the template, the TaqMan probe was degraded due to the 5'-3' polymerase activity and exonuclease activity of Taq DNA polymerase, then the separation of fluorescent

reporter and quencher enabled the fluorescent signal to be detected by the instrument. The ORF1ab gene of SARS-CoV-2 was detected qualitatively by the FAM channel, the N gene of SARS-CoV-2 was detected qualitatively by the JOE channel, and the E gene of SARS-CoV-2 was detected qualitatively by ROX channel, and the internal reference was detected by CY5 channel.[11] The analyzer calculates Cycle threshold (Ct) values for the amplification curves of gene ORF1ab, N, E, and both IC, respectively, and the results were reported positive or negative by the following criteria: If the Ct-value of gene ORF1ab (FAM), gene N (JOE) and gene E (ROX) < 36, the test result for gene ORF1ab or gene N will be positive; If the Ct-value of an IC (FAM, JOE, and ROX) is < 32, test result for an IC will be positive.

To determine the specificity and sensitivity of the CPA assay, 52 swab nasopharyngeal samples were tested with CPA, then the samples were performed again with rRT-PCR at Provincial Health Laboratory as SARS-CoV-2 PCR reference laboratory as the gold standard.

Software SPSS version 20 was used for all statistical analyses. The statistical analysis used diagnostic tests (sensitivity, specificity, positive predictive value, and negative predictive value) and the rank spearman correlation test.

This study was approved by the Ethics Committee for Health Studies R. Syamsudin, S.H. Hospital, Sukabumi with number 02/KEPK-RS/RSUD/I/2022.

RESULTS AND DISCUSSIONS

A Total of 52 nasopharyngeal swab samples were collected from patients who came to the emergency room of Palabuhan Ratu Hospital with COVID-19. The characteristics of the subjects are shown in Table 1.

Table 1 showed that the majority of subjects were females with age 34-50 years and positive SARS-CoV-2 patients predominated with females.

Cross priming amplification and rRT-PCR test were performed on a total of 52 clinical nasopharyngeal samples. The results of rRT-PCR and CPA testing were then stratified into Ct-value, as shown in Table 2.

Table 1. Characteristics of subjects

Variable	Subjects (n=44)	
	N	%
Gender, n (%)		
Male	22	43
Female	30	57
Age (year)		
0-16	3	6
17-33	16	31
34-50	22	42
51-67	10	19
≥ 68	1	2
SARS-CoV-2, n (%)		
Positive	34	65
Negative	18	35
Gender of positive SARS-CoV-2 patients, n (%)		
Male	13	38
Female	21	62

Table 2. rRT-PCR and CPA testing samples stratified into Ct-value

		CPA			
		Ct	Pos	Neg	Sum
rRT-PCR	Pos	0-25	20	2	22
		25-30	3	2	5
		31-35	4	3	7
		35-40	0	0	0
	Neg	Neg	1	17	18
	Sum		28	24	52

Table 2 showed that the majority of samples were on 0-25 Ct-values with 21 samples of CPA showing true positive and two samples showing false negative results. In addition, among the patient's samples with Ct-values of 31-40, there were 4 samples with true positive and 3 samples with false negative results.

Receiver Operating Curve (ROC) analysis Tt-value on COVID-19 patients is shown in Table 3.

The diagram ROC analysis of the Tt-value on COVID-19 patients is shown in Figure 1.

Figure 1 showed that the cut-off and Area Under Curve (AUC) of a total clinical sample Tt-value was 3.25 and 0.84, with a p-value <0.001, suggesting that CPA had a good validity for COVID-19 prediction with a sensitivity of 77% and specificity of 94%. The sensitivity of Tt-value increased with Ct-value <30 (82%) and Ct-value <25 (87%). However, the specificity remained unchanged (94%).

The correlation test was performed to determine the correlation strength between the Tt-value and with Ct-value. The correlation test results between Tt-value with Ct-value are shown in Table 4.

Table 3. The ROC analysis of the Tt-value on COVID-19 patients

Variable	ROC			
	AUC (95% CI)	p-value	Cut-off	Diagnostic Value
Tt-value	0.84 (0.72–0.96)	<0.001	≥3.25	Sensitivity: 77% Specificity: 94% PPV: 96% NPV: 71%
Tt-value with Ct -value <30	0.86 (0.74–0.99)	<0.001	≥3.75	Sensitivity: 82% Specificity: 94% PPV: 96% NPV: 81%
Tt-value with Ct -value<25	0.89 (0.76–1.00)	<0.001	≥3.75	Sensitivity: 87% Specificity: 94% PPV: 95% NPV: 89%

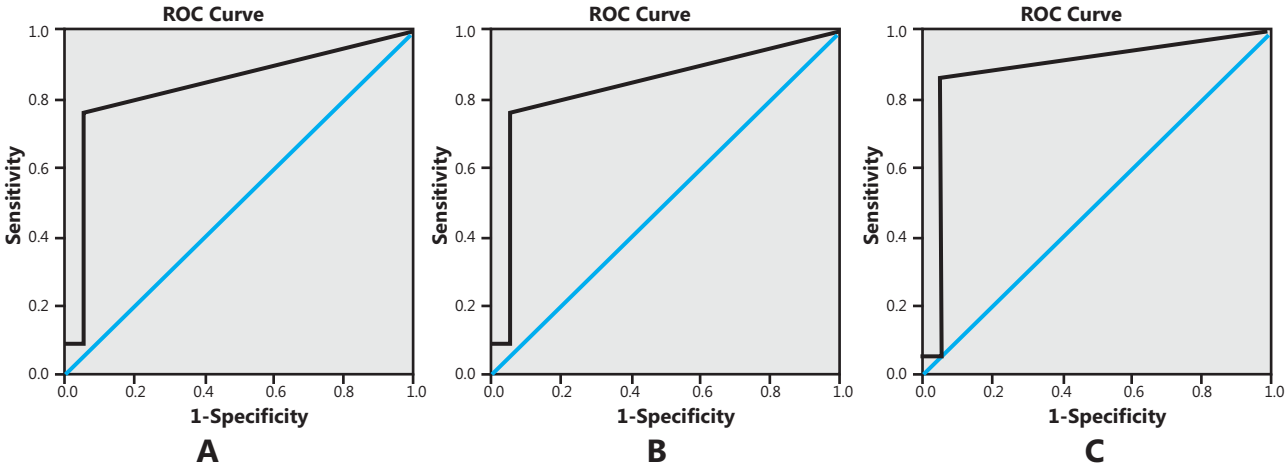

Figure 1. The ROC analysis of the Tt-value on patients suspected of COVID-19 (A) ROC Tt-value of COVID-19 Patients' clinical sample (B) ROC Tt -value on patients suspected with COVID-19 with Ct-value <30. (C) ROC Tt-value on COVID-19 patients with Ct-value<25

Table 4. Correlation between Tt-value and Ct-value

Variable	Median	Min-Max	p-value	Correlation Coefficient (r_s)
Ct -value (cycle)	29.3	21.3-37	<0.001	0.77
Tt-value (minute)	20.6	10.4-40		

Table 4 showed significant strong positive correlation between Tt-value and Ct-value (rs =0.77, p < 0.01).

The prevalence of COVID-19 between males and females is similar. However, males with COVID-19 are more at risk for worse outcomes and death, independent of age.[12] Based on the characteristics of subjects in this study, the majority of subjects were female with ages of 34-50 years (42%). Among positive confirmed SARS-CoV-2 patients, there was a higher number of females (56%), suggesting that females are more frequent to have SARS-CoV-2 infections. It was related to a lot of labor-age females at Sukabumi regency who were exposed to COVID-19 infection. The majority of the female patients were factory workers who were often clustered from COVID-19, thereby making them more vulnerable.[13]

The results in this study showed that the CPA has good validity for COVID-19 diagnosis with a sensitivity of 77%, specificity of 94%, and AUC of 84%, suggesting that CPA could give 84% true diagnosis of SARS-CoV-2 infections. The minimal cut-off AUC for the diagnostics test was 50%. Therefore, CPA could be used as rapid molecular testing for detecting SARS-CoV-2 viral RNA from pharyngeal swab specimens.[6,14]

The CPA had varying sensitivities with unchanged specificity. It had 82% sensitivity for Ct-value <30 and 87% sensitivity for Ct-value <25. For samples with Ct>30, the CPA assay was much less sensitive.[15,16] However, there is debate about, which Ct-value for a positive rRT-PCR result should be considered clinically relevant. Vogels *et al*. suggested that a Ct-value above 36 corresponds to less than 10 molecules of RNA.[17] It was concluded that the CPA assay would be suitable for identifying individuals with a high or moderate SARS-CoV-2 viral load. Contrastingly, for those with a low viral load (at the onset of illness or during later stages of the disease), the sensitivity of the CPA assay was insufficient to detect a SARS-CoV-2 infection.[14] The low sensitivity for low viral load with Ct-value >30 was correlated with the different Limits of Detection (LoD) between CPA and rRT-PCR.[15] The CPA has a higher LoD (103 Copies) than rRT-PCR (10 copies).[10,18]

The Tt-value was analyzed for quantification of the CPA. The correlation between Tt-value and Ct-value was determined to evaluate Tt-value performance. Correlation test in this study showed significant strong positive correlation between Tt-value and Ct-value (rs =0.77, p <0.01). The time threshold was concluded as quantification for CPA, which was analog with Ct-value at rRT-PCR.

CONCLUSIONS AND SUGGESTIONS

The CPA has good validity for COVID-19 diagnostic with sensitivity, specificity, PPV, and NPV of 77%, 94%, 96%, and 71%, respectively. The CPA could be used as rapid molecular testing for detecting SARS-CoV-2 viral RNA in RNA isolated from pharyngeal swab specimens.

The limitation of this study is a small number of samples, which is affecting the validity of CPA. It is recommended to use a larger sample number for future studies to assess better validity, and performance of CPA.

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