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Development and Optimization of SARS-CoV-2-Specific Primers for Accurate Diagnosis: A Case Study in West Sumatra, Indonesia

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Abstract

Background: In 2022, new cases of Covid-19 emerged, including the Omicron variant which is classified as a variant of concern (VOC). West Sumatra is one of the top ten provinces with the highest number of cases in Indonesia. This study aimed to design specific primers and optimize the PCR method that can be used for accurate detection, specifically for SARS-CoV-2 circulating in West Sumatra, Indonesia.

Methods: This study used an in silico approach, using whole genome sequencing (WGS) data available at the global initiative on sharing avian influenza data (GISAID), and employing the Geneious Prime application which confirmed samples collected from Padang, West Sumatra, and from Jakarta, Bogor, Depok, Tangerang, Bekasi (Jabodetabek) serving as comparative sample tests. Technology development was supported by bioinformatics testing, laboratory testing, and validation methods, involving gene mining, sequence alignment, and primer design. Laboratory tests and validation included viral genomes extraction and cDNA synthesis, polymerase chain reaction (PCR) testing, and results analysis.

Results: Three sets of optimal primer candidates amplified the coveted target gene was discovered, specifically, the S gene of the receptor binding domain (RBD) region.

Conclusions: The primers designed through a consensus between the complete genome of the SARS-CoV-2 isolate Wuhan-Hu-1 and the WGS of the Omicron variant in Padang, West Sumatra, have successfully detected the SARS-CoV-2 virus variant in the region. The most effective temperature optimization results were achieved by testing three primer products on samples from Padang and Jabodetabek. It has significance as a valuable diagnostic tool in the primer form.

Keywords: Omicron variant, Bioinformatics, PCR testing, Primer design, SARS-CoV-2

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Introduction

Coronavirus Disease 2019 (COVID-19) is a disease caused by a single-stranded RNA virus, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). This disease first appeared in Wuhan, China in December 2019. SARS-CoV-2 has a higher transmission rate than SARS and middle east respiratory syndrome (MERS).[1]

The characteristics of the SARS-CoV-2 virus have structural proteins, namely membrane proteins (protein M), Envelope proteins (protein E), and Nucleocapsid proteins (protein N). The protein S of the coronavirus envelope is targeted because this protein is the gateway for the virus to enter the host cell. The protein S has a structure in the form of spikes which become a place for viruses to attach to host cell receptors and enter host cells, so protein S greatly determines this virus's malignancy level.[2] They entry receptor for SARS-CoV-2

and the original SARS-CoV (referred to here as SARS-CoV-1) is the human cell surface protein angiotensin converting enzyme 2 (ACE2). The spike receptor binding domain (RBD) of these two viruses has high affinity to bind with ACE2.[3]

To address the problem, it is necessary to develop an accurate, faster, and efficient PCR-based method, as well as to identify specific gene regions on segments of the viral genome that can be precisely used as molecular detection markers.[4] Generally, the diagnosis of SARS-CoV-2 is performed using two methods: rapid tests and Polymerase Chain Reaction (PCR), which aim to detect the spread of coronavirus in Asia in general and Indonesia in particular, especially in middle and developing countries.[5] In carrying out a PCR test, the primer is a crucial component since it can specifically target the DNA to be amplified, making the PCR method highly accurate. Designing new primers is a preferable option over using existing ones. The advantage of designing new primers is that researchers can ensure the performance of the test to optimize the method and avoid process failures or experimental errors.[6]

Therefore, this study delves deeper into the use of PCR-based detection systems by reviewing the utilization of appropriate target sequences and more precise primer designs. The study commenced with the design of specific primers for SARS-CoV-2 in a particular sample from Padang, West Sumatra, Indonesia followed by the optimization of a PCR method that is specific to SARS-CoV-2 Padang, West Sumatra, Indonesia. The study aimed to ensure reliable test outcomes during the developmental phase, thereby facilitating the practical application of these findings, particularly in SARS-CoV-2 detection efforts.

Methods

SARS-CoV-2 viral transport media (VTM) samples were collected from the Diagnostic and Integrated Research Laboratory of Infectious Diseases, Faculty of Medicine, Universitas Andalas in Padang, West Sumatra. The samples were obtained from patients diagnosed with COVID-19, with a CT value of 21.33. The whole genome sequencing analysis had confirmed that this sample was of the Omicron variant. The genome sequence of SARS-CoV-2 (nc_045512) was utilized for in silico testing. Specifically, the protein part of the receptor binding domain (RBD) domain, with a length of 669 base pairs and a nucleotide sequence (21563..25384), was targeted using the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/).

The Omicron variant of SARS-CoV-2 from Padang, West Sumatra was obtained from the Global Initiative On Sharing Avian Influenza Data (GISAID; https://gisaid.org/). Subsequently, primers were designed using Geneious Prime software (biomatters ltd., version 2022.2.2). A consensus was obtained from multiple alignments between the whole genome sequence from Wuhan taken from Genbank NCBI and all sequences of the SARS-CoV-2 variant of Omicron Padang, West Sumatra taken from GISAID and one sample from the area around the capital city of Indonesia, Jakarta, known as Jakarta, Bogor, Depok, Tangerang, and Bekasi (Jabodetabek) as a comparison of sample testing (Figure 1). The specificity of the designed primers was examined using Geneious Prime software to ensure that they properly bound and matched the template DNA sequence.

The process of extracting the viral genome was conducted on samples suspected of containing the virus (obtained from swab results). The extraction was carried out in a Laboratory Biosafety Level 2 facility of National Research and Innovation Agency Republic of Indonesia (*Badan Riset dan Inovasi Nasional*, BRIN) and RNA from the SARS-CoV-2 virus was extracted using a kit-based method. In this research, cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific), and the steps involved in the process were explained in the kit manual. The Polymerase Chain Reaction (PCR) in this study was optimized using the Thermo Scientific DreamTaq DNA Polymerase, with the processing steps described in the kit. The PCR program was set as follows: an initial denaturation at 98°C for 30 seconds, followed by 35 cycles consisting of a denaturation step at 98°C for 5 seconds, an annealing stage at a gradient temperature of 50–61°C for 15 seconds, and an elongation stage at 72°C for 15 seconds. The final elongation stage, which only cycled once, was at 72°C for 5 minutes. PCR results were visualized by electrophoresis of a 2% agarose gel prepared previously by dissolving 1 g of agarose in 50 ml of TAE 1x.

The sequencing method employed was Sanger Sequencing. The sequencing results were analyzed using the Bioedit application, and the species identification was conducted using BLASTn on the NCBI Genbank website online. The purpose of sequencing analysis is

Figure 1 Sampling Locations for the SARS-CoV-2 Omicron Variant (Padang, West Sumatra and the Jakarta, Bogor, Depok, Tangerang, and Bekasi Area

to determine the nucleotide sequence of the amplified DNA and ensure the identification of the target gene species.[7]

Results

The results of multiple alignments led to the identification of three pairs of the best primer candidates (Table 1). It was observed that the attachment between the primers and the target gene was appropriate, as proven in silico (Table 2). Based on the results of the in silico test, all primers appeared to bind to the intended target gene, resulting in appropriate amplicon lengths. To optimized the annealing temperature, a temperature gradient was performed on the three primer pairs that had been ordered. The gradient temperature range used was 52–61°C, as indicated by the vendor's Tm (Figure 2). The best temperature optimization results were obtained from testing three primer products on one sample from Padang, West Sumatra, and one sample from Jabodetabek (Figure 3) as a comparison of sample testing.

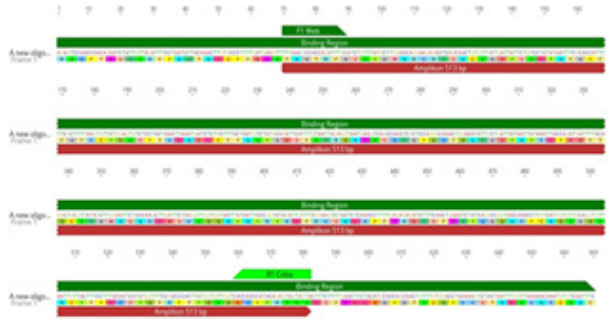
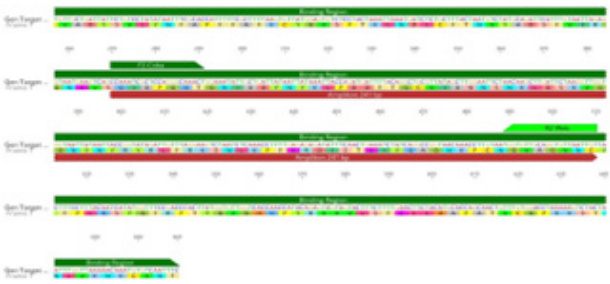
PCR products from the Padang, West Sumatra, sample were sequenced using primer pairs F1W/R1C, F2C/R2W, and F2Co/R1Co. The sequencing results produced DNA bands with sizes of 513 bp, 241 bp, and 313 bp. The DNA bands were thick enough and suitable for sequencing. Sequencing using the three primer pairs produced DNA sequences measuring 462 bp, 232 bp, and 314 bp, which were then combined. The results of the sequencing data analysis are presented in Table 3.

The data presented had shown that the optimization tests on primer 1, primer 2, and primer 3 resulted in all temperature gradients being amplified with the appropriate amplicon size. The thickest band in primer 1, with an amplicon length of 513 bp, was observed at 54.4ºC (C). The thickest band in primer 2, with an amplicon length of 241 bp, was observed at 60ºC (D). The thickest band in primer 3, with an amplicon length of 313 bp, was observed at

Table 1 Primer Candidate Characteristics

Primer Name	Primer Pair	Primer Sequence	Product Lenght	Language Lenght	%GC	Melting Point Tm (°C)
F1 Web	Forward	5' TTTAACGCCACCAGATTTGC 3'	513 bp	20	45.0	57.3
R1 Coba	Reverse	5' TACTACTACTCTGTATGGTTGGTG 3'		24	41.7	57.0
F2 Coba	Forward	5' CCAAATCGCTCCAGGGCAAACT 3'	241 bp	22	54.5	63.7
R2 Web	Reverse	5' ACAATTAACACCTGCAACACCA 3'		22	40.9	59.0
F2 Coba	Forward	5' CCAAATCGCTCCAGGGCAAACT 3'	313 bp	22	54.5	63.7
R1 Coba	Reverse	5' TACTACTACTCTGTATGGTTGGTG 3'		24	41.7	57.0

Table 2 Primer Pair Amplifier 1, Primer 2, and Primer 3

Primer Name	Primer Amplification
Primer 1 (F1 Web & R1 Coba)	
Primer 2 (F2 Coba & R2 Web)	
Primer 3 (F2 Coba & R1 Coba)	

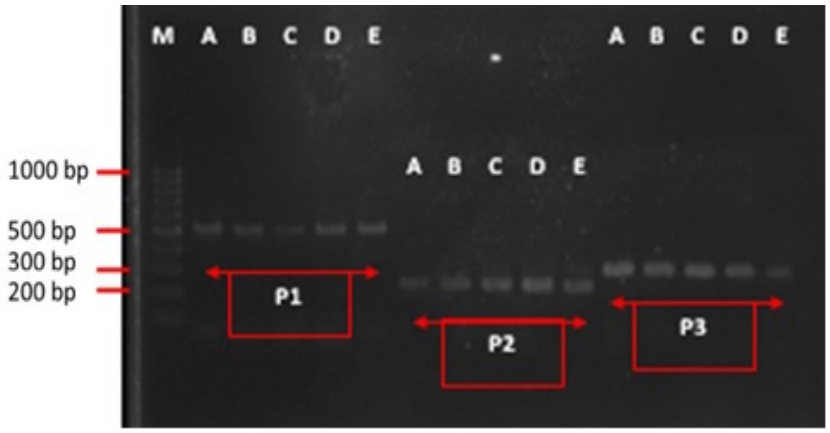
a temperature of 55.7ºC (C). The thicker the DNA bands appear, the more DNA templates were amplified. Thus, the optimum annealing temperature for primer 1 was 54.4ºC, for primer 2 was 60ºC, and for primer 3 was 55.7ºC.

After contiguous sequencing from primer 1, the data had a length of 462 bp, primer 2 had a length of 230 bp, and primer 3 had a length of 316 bp (Table 3). All three primer pairs correctly identified the severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2 species with 100% identity, and no other species were identified.

Discussion

The design of primers plays a crucial role in the amplification of DNA segments. Experiment in silico is commonly used to ensure good primer design. Primers are utilized in the polymerase chain reaction (PCR) technique, where they hybridize to a DNA template and dictate the sites to be amplified. They serve as the attachment points for the DNA polymerase to amplify the target gene.[9] The ideal candidate primers should have a base length between 18–28 bp, as these have been shown to produce the best amplification. Longer primers provide greater specificity but lead to fewer amplicons due to a longer elongation process. Additionally, excessively long primers do not increase specificity but can lead to higher production costs.[10] The percentage of G and C (%GC) in a primer is crucial. The optimal GC percentage range from 40–60%. If the %GC value is too low, the primer may not bind selectively to the target, whereas if it is too high, non-specific results may be obtained.[11]

Consensus sequence data is an important step before proceeding with the main design process. The consensus sequence resulting

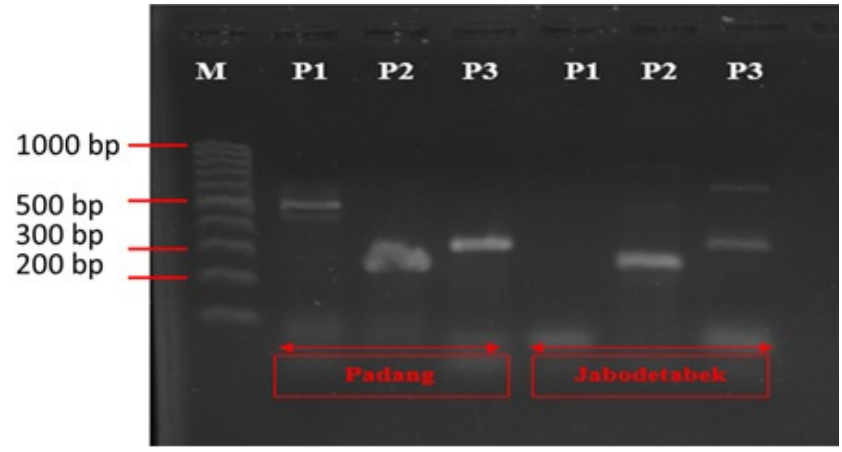


M: 100 bp ladder	Gradient Temp (°C):	Gradient Temp (°C):	Gradient Temp (°C):
P1: Primer 1	P1	P2	P3
P2: Primer 2	A: 52.0	A: 54.0	A: 52.0
P3: Primer 3	B: 53.0	B: 55.0	B: 53.3
	C: 54.4	C: 58.0	C: 55.7
	D: 55.0	D: 60.0	D: 60.4
	E: 56.0	E: 60.5	E: 61.0

Figure 2 Gradient PCR Test Results using Primer 1, Primer 2, and Primer 3
Note: The thicker the DNA bands appear, the more DNA templates are amplified.[8]

from the alignment process is an important factor in comparative analysis studies.[10] The sequence data obtained showed good similarity in all sequences. This can be seen from the conserved sequences without any gaps between sequences. Consensus sequences are often used as templates to search for similar sequences[13] to design a protein with a high balance and to design a primer.[14]

The methodology described in this study ensured 100% coverage of the S gene. The research began with the design of primers that followed good primer characteristics. Primer pairs 1, 2, and 3 were designed to meet the criteria for good primer design. It was ensured that forward and reverse primers did not



M: 100 bp ladder	Annealing Temp (°C):
P1: Primer 1	P1: 54.4
P2: Primer 2	P2: 60.0
P3: Primer 3	P3: 55.7

Figure 3 Primer Test Results on Samples from Padang and Jakarta, Bogor, Depok, Tangerang, and Bekasi (Jabodetabek)

Table 3 Sequencing and Blast Results

Primer Name	Contig Result	Blastn Results (Identity)
Primer 1 (F1 Web & R1 Coba)	>Contig1 TTTTAACGCC ACCAGATTTGCATCTGTTTATGCTTGGAACAGGAAGAGAATCAGCAA CTGTGTTGCTGATTATTCTGTCCTATATAATTTCGCACCATTTTTCGCTTTTAAGTGTT ATGG AGTGTCTCCTACTAAATTAAATGATCTCTGCTTTACTAATGTCTATGCAGATTCATTTG TAATTAG AGGTAATGAAGTCAGCCAAATCGCTCCAGGGCAAACTGGAAATATTGCTGA TTATAATTATAAATTACCAGATGATTTTACAGGCTGCGTTATAGCTTGGAATTCTAACA AGCTTGATTCTAAGGTTGGTGGTAATTATAATTACCGGTATAGATTGTTTAGGAAGTC TAATCTCAAACCTTTTGAGAGAGATATTTCAACTGAAATCTATCAGGCCGGTAACAAA CCTTGTAATGGTGTTGCAGGTGTTAATTGTTACTTTCCTTTACAATCATA	100%
Primer 2 (F2 Coba & R2 Web)	>Contig1 CCAAATCGCTCCAGGGCAAACTGGAAATATTGCTGATTATAATTATAAATTACCAGAT GATTTTACAGGCTGCGTTATAGCTTGGAATTCTAACAAGCTTGATTCTAAGGTTGGTG GTAATTATAATTACCGGTATAGATTGTTTAGGAAGTCTAATCTCAAACCTTTTGAGAG ATATTTCAACTGAAATCTATCAGGCCGGTAACAAACCTTGTAATGGTGTTGCAG	100%
Primer 3 (F2 Coba & R1 Coba)	>Contig1 TCCAAATCGCTCCAGGGCAAACTGGAAATATTGCTGATTATAATTATAAATTACCAGAG ATTTTACAGGCTGCGTTATAGCTTGGAATTCTAACAAGCTTGATTCTAAGGTTGGTGG TA ATTATAATTACCGGTATAGATTGTTTAGGAAGTCTAATCTCAAACCTTTTGAGAGAGAT ATTTCAACTGAAATCTATCAGGCCGGTAACAAACCTTGTAATGGTGTTGCAGGTGTTA ATTGTTACTTTCCTTTACAATCATATGGTTTCCGACCCACTTATGGTGTTGGTCACCAA CCATACAGAGTAGTAGTAAC	100%

have secondary structures such as hairpins or self-dimers, which can prevent the designed primer from binding to the DNA template.[15] In addition, the primers were tested in silico using the Geneious Prime application, and the results showed that the primers had attached to the desired target gene, the S gene for the receptor binding domain (RBD) region.[16] The size of the amplicon of each primer was in accordance with the design results, with an amplicon length of 513 bp for the 1st primer pair, 241 bp for the 2nd primer pair, and 313 bp for the 3rd primer pair. The DNA template used in this study was extracted from RNA from the SARS-CoV-2 Viral Transport Media (VTM) variant of Omicron Padang, West Sumatra. The purity and concentration of the DNA samples were critical factors for successful DNA amplification. The samples had a high purity and concentration of 3666.2 ng/µL and purity (260/280) 1.76, respectively, which were determined using a nanodrop.[17,18]

The optimization of annealing temperature is a crucial factor for successful DNA amplification, as annealing is a critical step in PCR where the primer binds to the target DNA. If the temperature is too low, the primer will stick non-specifically, and if it is too high, the amplification process can fail.[19,20] Primer temperature optimization was optimized using the principle of singleplex PCR. Singleplex PCR is a method of detecting target sequences in a PCR reaction. This PCR test is a quick and easy approach and is suitable for diagnosis and large-scale epidemiological studies.[21] If the results of primer annealing temperature optimization are the same, the primers can be used as molecular diagnostic test tools. To achieve this goal, the PCR reaction used should be multiplex PCR because it can target several genes simultaneously in one reaction.[22] It is necessary to have same annealing temperature in multiplex PCR.

The results of PCR re-validation for sequencing, utilizing the same sample as that used for determining the optimum annealing temperatures for the primers, provide valuable insights into the performance of the designed primers. It was observed that primer 1 exhibited an optimal annealing temperature of 54.4°C, primer 2 at 60°C, and primer 3 at 55.7°C. These findings underscore the importance of precisely determining annealing temperatures for each primer to

ensure efficient amplification of the target gene. The thicker the DNA bands appear, the more DNA templates are amplified.[8]

Furthermore, the successful detection of the target sample when the designed primers were tested on a Jabodetabek sample with a CT value of 37.15 is particularly noteworthy. This indicates the robustness and reliability of the primer set across different sample types and CT values, further validating their efficacy in detecting the SARS-CoV-2 virus.

These results suggest that the designed primers are capable of effectively detecting the target gene across a range of annealing temperatures and sample conditions, thus demonstrating their potential utility as a diagnostic tool for SARS-CoV-2 detection. Moreover, the successful detection of the target sample in a Jabodetabek sample emphasizes the applicability of these primers beyond the specific geographic region of Padang, West Sumatra, enhancing their versatility and utility in broader diagnostic settings.

The study is subject to certain limitations due to the restricted availability of SARS-CoV-2 samples from provinces beyond West Sumatra and wildtype samples of the omicron variant from Wuhan. Consequently, it is unable to directly compare the specific primers designed for Padang, West Sumatra, with the reference sequence of the omicron variant from Wuhan.

In conclusion, the primers develop in this study, which are based on a consensus between the complete genome of the SARS-CoV-2 isolate Wuhan-hu-1 and the WGS sequence of the SARS-CoV-2 variant Omicron in Padang, West Sumatra, have demonstrated their efficacy in detecting the omicron variant padang, as well as in samples from Jabodetabek. Consequently, these primers hold promise as valuable diagnostic tools in primer form, facilitating the accurate detection of the SARS-CoV-2 virus.

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