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Development of an in-house primer design: Nested polymerase chain reaction for coronavirus disease 2019 detection based on open reading frame 1a/b and spike

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Abstract

Background and Aim: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has infected more than 1 million people and caused more than 100,000 deaths in Indonesia. This condition was augmented by a less advanced health system, especially in providing diagnostic facilities for the novel coronavirus, and the high mutation rate of the novel coronavirus, which may promote the generation of specific strains in Indonesia. This study aimed to propose a specific primer (in-house primer) toward open reading frame 1a/b (*ORF1ab)* and the spike protein gene of SARS-CoV-2 to detect SARS-CoV-2 and to analyze the presence of mutations.

Materials and Methods: One hundred and nine samples were collected from patients in Malang, East Java, Indonesia. The samples were extracted using QIAamp viral RNA kits. The in-house primer was designed using Clone Manager 9.0 and amplified using nested polymerase chain reaction (PCR). Then, the amplicon was analyzed through sequencing. The detection results were compared with those obtained using the quantitative PCR (qPCR).

Results: Nested PCR was 74.3% positive, whereas qPCR was 45.9% positive. Furthermore, sequencing analysis of the amplicon revealed the mutation at locations T3187C, T2889C/T, G3189T (spike), and C364T (*ORF1ab*). The sensitivity and specificity of nested PCR were 92.6% and 43.6%, respectively. This result indicated that the in-house primer performed well at screening.

Conclusion: In-house primers could detect SARS-CoV-2 and mutations in samples from Malang, East Java, Indonesia. In the future, this method could be recommended as a screening tool for monitoring SARS-CoV-2 infection.

Keywords: nested polymerase chain reaction, open reading frame 1a/b, primer, severe acute respiratory syndrome coronavirus 2, spike.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has infected more than 1 million people and caused more than 100,000 deaths in Indonesia [1, 2]. In January 2022, there was the highest number of active Coronavirus disease 2019 (COVID-19) cases in East Java, Indonesia, specifically in Malang City, with 205 cases [2]. Indonesia is affected by the pandemic of these viruses, especially Malang. The COVID-19 variants detected in Indonesia included the sub-variants Omicron JN.1.1 and JN.1.39 [2, 3]. However, there are also new variants, namely, KP.1 and KP.2, which have been found in Singapore [2, 3]. This is crucial to be aware of, as there have been suspected cases in Yogyakarta, with a mortality rate of 0.00/100,000 people and a positive response capacity indicator of 0.88%, with an incidence rate of 0.27/100,000 people [3]. The rapid spread of cities in Indonesia has had a serious impact. This condition is augmented by less advanced health systems, especially those providing the best facilities against the novel coronavirus and the high mutation rate of the novel coronavirus, which may promote the generation of specific strains in Indonesia [1–4]. For this reason, Indonesia have focused on local strains of novel coronaviruses.

Polymerase chain reaction (PCR) is an acknowledged molecular method for rapidly diagnosing these diseases despite the less favorable viral culture. Using this method, a medical worker can detect the virus by amplifying the genetic material from the RNA

samples of the patient [4]. The PCR process requires a specific oligonucleotide called a primer, which hybridizes with the genetic material of the virus [5]. However, most primers were imported from many developed countries like the United States of America, China, and many European countries, and these primers might not have sufficient coverage to detect some virus mutations in certain local strains of the novel coronavirus [5, 6].

The development of detection methods has also been reported since the virus rapidly spread worldwide through the Centers for Disease Control and Prevention (CDC), such as the Chinese CDC, the American CDC, and other private companies. The majority of detection using quantitative PCR (qPCR) testing has become the current standard diagnostic method for COVID-19; however, high demand could lead to shortages, making it necessary to have an alternative [7]. Endpoint RT-PCR could be such an alternative. The cost of PCR is lower and is feasible for laboratories that do not have RT-PCR equipment. Among the various endpoint PCR strategies, the significance of studies used nested PCR. Nested PCR is highly sensitive and specific and uses two sets of primers (first round and second round). The development of detection using nested PCR is important, unlike tool methods, such as qPCR, which are not optimal for detecting SARS-CoV-2 mutations, especially in the Indonesian region.

A method for this detection is the development of region-specific detection methods, particularly for strains found in Indonesia, particularly in the Malang area. Until now, few quick, effective, inexpensive, and easy-to-apply detection methods exist in Malang, East Java, Indonesia. This study aimed to propose a specific primer (in-house primer) toward specific locations on open reading frame 1a/b (*ORF1ab*) and spikes of the SARS-CoV-2 virus. In addition to rapid detection, it is also hoped that new mutations can be found, which can later be reported and brought to the attention of policymakers for further investigation.

Materials and Methods

Ethical approval and Informed consent

This research was reviewed and approved by the Ethical Review Board of the Faculty of Medicine, University of Brawijaya (No. 63/EC/KEPK/02/2021). We used leftover RNA of each patient with COVID-19 symptoms from hospitals around Malang who came to RSUB, which was approved by the ethical clearance committee. The RNA was isolated from the patient blood samples which were processed routinely at RSUB, Malang, East Java, Indonesia. Verbal consent was obtained from each patient/relative of the patient for sample collection.

Study period and location

This study was conducted from July 2020 to July 2021 at Brawijaya University Hospital (RSUB).

Sample collection

We used leftover RNA of each patients with COVID-19 symptoms in a hospital in Malang, East Java, Indonesia. RNA was extracted from them at the Virology Laboratory in Malang, East Java, Indonesia. These samples were selected randomly and classified as positive (54) and negative (55) according to previous RT-PCR results using commercial primers to detect SARS-CoV-2. Patient samples were tested with q-PCR and showed positive results. Samples from nasopharyngeal or oropharyngeal samples were collected using the QIAamp RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNA was stored at −80°C. The obtained RNA samples were then subjected to RNA quality testing using a nanodrop at an absorbance wavelength of 260/280. The results were within the range of 1.8–2.

Primer design

The primer pairs were designed using Clone Manager 9.0 (Scientific & Educational Software, North Carolina, USA) based on the whole genome of the Wuhan strain SARS-CoV-2. Our primer pair used the spike and *ORF1ab* as the target of the amplicon in nested PCR. The primer pairs for the spike target were as follows: the first round: SKWN_WH1S_F1 (forward) and SKWN_WH1S_R1 (reverse) and continue for the second round: SKWN_WH1S_F2 (forward) and SKWN_WH1S_R2 (reverse). The primer pairs for the *ORF1ab* target were as follows: First-round SKWN_WH1_F183F1 (forward) and SKWN_WH1_R578R1 (reverse) and second-round SKWN_WH1_F221F2 (forward) and SKWN_WH1_R559R2 (reverse). Our primer pairs have been registered as a patent (patent number: P0022021110043).

Nested PCR

The RNA samples were converted to complementary DNA (cDNA) using a Goscript reverse transcription system kit (Promega Corporation, Wisconsin, USA) and the protocol. The cDNA samples were collected and briefly amplified by nested PCR using the primers F183F1 and R578R1 in the first round. The temperature for the amplification methods was as follows: The initial step at 94°C for 1 min, 94°C for 30 s, 62°C for 30 s, 68°C for 30 s, and the final step at 68°C for 5 min for 30 cycles. The amplicon was collected and briefly amplified for the second round using F221F2 (forward) and R559R2 (reverse). The second-round amplification consisted of an initial step at 94°C for 1 min, 94°C for 30 s, 45°C for 30 s, 68°C for 30 s, and a final step at 68°C for 5 min for 35 cycles.

Sequencing analysis

The amplicon was analyzed for sequencing. The amplicon was sequenced using 1st BASE Sequencing INT (First BASE Laboratories Sdn Bhd, Selangor, Malaysia).

In-house primer analysis

To evaluate the performance of the recently developed in-house primer, we calculated its

sensitivity, specificity, positive predictive value, and negative predictive value using table 2 × 2 (Two-fold).

Results

The total number of RNA samples collected was 109. The samples were amplified using nested PCR to detect SARS-CoV-2. Our research used an in-house primer design using clone manager 9.0 (scied.com) as the spike vector and *ORF1ab* as the target vector. New primers with optimum concentrations were obtained using the designated PCR primers used in our experiments. The novel primer met the criteria for a good primer. The characteristics of the recent in-house primers are shown in Table-1.

Several main criteria, including the primer length, melting temperature, and guanine-cytosine (GC) content, must be considered when designing PCR products. Our novel primer length was 22–27 nucleotides long. The annealing temperature ranges from 45°C to 62°C. The GC content in our primer ranged from 36% to 56%. Our in-house primer is distinct from commercially available primers based on criteria such as length, melting temperature, and GC content. A commercial primer for q-PCR is used with a probe, whereas an in-house primer design may make it easier to apply samples with high specificity and sensitivity. With this distinct structure, we aimed to increase the yield during amplification. Our primer generated a longer amplicon than the commercial primer. The ability of the primers to detect positive samples as positive (specificity) and negative samples as negative (sensitivity) compared with commercially available primers is shown in Table-2.

Despite the positive results obtained using an in-house primer design, sequencing revealed mutations in the nucleotide sequences in both the spike region and the *ORF1ab* region. Mutation at position 3189 from guanine to thymine, a change from thymine to cytosine at position 3187, and a change from thymine to cytosine/thymine at position 2889 in the spike region. Additionally, a change in *ORF1ab* from cytosine to thymine at position 364. The mutation for G3189T has translated into an amino acid and has changed to L1063F (leucine to phenylalanine) at the location of the full-length spike. The mutation at T3187C in the spike region did not alter the amino acid content of leucine. These values are also the same for T2889C/T for Valine. Our findings indicate that the mutation is associated with single nucleotide polymorphism (SNP) at the spike region, as mentioned above. The SNP was found in the spike region and is categorized as synonymous with an SNP. The C363T mutation in the *ORF1ab* region also changes the amino acid F122L (phenylalanine to leucine).

Discussion

The design process of PCR primers should be thoroughly considered because a minor defect in the primer can affect the yield of the amplification process [4]. The optimal in-house primer could be an adequate diagnostic tool for detecting the virus. The primer pairs have 22–27 nucleotide sequences, all meeting the ideal PCR primer criteria [4, 8, 9]. These primer pairs designed for the spike region have amplicon targets of 864 and 585 bp for the first round and 585 bp for the second round.

The effectiveness of the specific primer design was determined by the ability of the primer to specifically amplify the desired gene target. These assessments assess diagnostic tools' sensitivity, specificity, and predictive value. Here, our in-house primers showed strong performance in detecting the genetic material of SARS-CoV-2. The specificity and sensitivity of the in-house primers were 92.6% and 43.6%, respectively (Table-2), indicating that the in-house primers were useful for screening. High specificity and relatively low sensitivity values are suitable for ruling out negatively tested samples [10–12].

The in-house primers' predictive values were 61.7% and 85.7% for positive and negative predictive values, respectively. The in-house primer's significant negative predictive value indicated a low disease prevalence. The disease prevalence over a certain period affects the negative predictive value. Hence, the negative predictive value decreases as the disease prevalence increases [12, 13].

Furthermore, as mentioned above, the design of the primer pair is important for meeting the criteria for

Table-1: Characteristics of recent in-house primers.

Primer name	Primer sequence	Annealing temperature	Absorbance
Orf1ab 1st round			
SKWN_WH1_F183	5' GCCTCAACTTGAACAGCCCTATGTG 3′	62°C	32.6 µg/mL
SKWN_WH1_R578	5' GCACTCAAGAGGGTAGCCATCAG 3′		30.7 µg/mL
Orf1ab 2nd round			
SKWN_WH1_F221	5' CGGATGCTCGAACTGCACCTCATGG 3'	55°C	33.0 µg/mL
SKWN_WH1_R559	5' CGGATGCTCGAACTGCACCTCATGG 3′		31.5 µg/mL
Spike 1st round			
SKWN_WH1S_F	5' GGACCTTTGGTGCAGGTGCTGCATTAC 3′	55°C	33.2 µg/mL
SKWN_WH1S_R	5' CATAAACTGTGTTGTTGACAATTCC 3′		31.8 µg/mL
Spike 2nd round			
SKWN_WH1S_F2	5' GGTATTGGAGTTACACAGAATG 3'	45°C	29.9 µg/mL
SKWN_WH1S_R2	5' GTGCCATTTGAAACAAAGACAC 3'		30.3 µg/mL

Table-2: Diagnostic analysis.

In-house primers using nested PCR	Commercial primers using qRT-PCR		
	Positive sample	Negative sample	Total
Positively detected	50	31	81
Negatively detected	4	24	28
Total	54	55	109

Sensitivity=92.6%; Specificity=43.6%; PPV=61.7%; NPV=85.7%, RT-PCR=Real-time polymerase chain reaction, PPV=Positive predictive value, NPV=Negative predictive value

detecting the target [13–15]. As mentioned before, our study results for the spike region showed the mutation and SNP. Mutation in G3189T and change in the amino L1063F (leucine to phenylalanine). Mutation at position 3189 from guanine to thymine and amino acid change at position 1063 from leucine to phenylalanine. Leucine is a unique amino acid that plays a key role in regulating protein synthesis and can initiate translation [15, 16]. On the other hand, phenylalanine can play a critical regulatory role in cytoplasmic virion environments [16, 17].

SNP has similarities with the multiple nucleotide variations known as synonymous SNPs, which indicates that changes in nucleotide arrangement were also obtained. The importance of discovery of this mutation could be used in studying genetic variations and could be developed into a detection marker. The obtained gene mutations are important for learning, especially regarding their role in infection. The spike region is an important determinant of the SARS-CoV-2 virus [18]. This function also mediates viral attachment to host cell surface receptors and facilitates fusion between the virus and host cell membrane. It is also the main target of neutralizing antibodies produced after infection by SARS-CoV-2 [19–21]. Another finding was that the mutation at the ORF1ab location was C363T, which could change the amino acid F122L. As described by Charles *et al*. [18] and Harvey *et al*. [19], the amino acid phenylalanine could play a role in the cytoplasmic virion environment. Leucine has a role in protein synthesis and initiation for translating proteins [15–17].

As mentioned above, genome mutation is a common phenomenon in viruses to repair damage in the genetic material so that the virus can survive inside the host cells [21, 22]. Viral mutation capabilities can be observed in many viruses, such as HIV, influenza, hepatitis, and coronavirus [22–25]. In coronavirus, the mutation rate is slower than that of other viruses such as HIV or influenza. The mutation in coronavirus mainly occurs in the spike glycoprotein, which serves as a ligand in initiating infection [26]. In this study, the amplicon resulting from the amplification was sequenced. Following gene sequencing, we detected four mutations in the spike protein and one mutation in ORF1ab. The discovery of the mutation in the spike glycoprotein and *ORF1ab* genes is in concordance with a study by Tang *et al.* [26]. and this information could be of merit for developing COVID-19 vaccines. However, nested PCR is not feasible as a routine test because it is time-consuming and has a high risk of cross-contamination [26, 27]. One-step nested PCR, in which two successive rounds of amplification are performed in the same reaction tube, is as sensitive as two-step nested PCR. Therefore, we propose using one-step nested RT-PCR.

The results showed high sensitivity but still low specificity. It is suggested that further studies should be conducted to improve the specificity with a wider global sample range.

Conclusion

The dynamic features of SARS-CoV-2 and the rapid spread of COVID-19 have raised public attention and led to many novel studies to identify the best approach to handle this disease. An effective and reliable diagnostic tool is still in demand for the local variant of SARS-CoV-2 and to act as a source of knowledge for developing a novel vaccine. Hence, in this study, we demonstrated the development of a novel primer that was structurally good, had a strong performance in detecting local variants of SARS-CoV-2, and was capable of detecting local mutations of SARS-CoV-2. This study presents data on SARS-CoV-2 infection in the Malang area and will be used in further research in other regions of Indonesia. The discovery of SNPs and synonymous SNPs could be developed into detection markers.

Authors' Contributions

WYP and AW: Collected the samples. SW, SK, SA, DRR, RA, AT, and WYP: Analyzed the data and results. SK, SW, and SA: Designed the experiments. SK, DRR, RA, AT, and WYP: Performed experiments and analysis. All authors have drafted, read, reviewed, and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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