The Employment of Real-Time Polymerase Chain Reaction Using Species-Specific Primer Targeting on D-Loop Mitochondria for Identification of Porcine Gelatin in Soft Candy

Nina Salamah[1,2,3], Yuny Erwanto[4,5], Sudibyo Martono[1], and Abdul Rohman[1,5*]

[1]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia*

[2]*Department of Analytical Chemistry, Faculty of Pharmacy, Universitas Ahmad Dahlan, Jl. Prof Soepomo, Janturan Yogyakarta 55164, Indonesia*

[3]*Ahmad Dahlan Halal Center, Universitas Ahmad Dahlan, Jl. Prof Soepomo, Janturan Yogyakarta 55164, Indonesia*

[4]*Division of Animal Products Technology, Faculty of Animal Science, Universitas Gadjah Mada, Jl. Fauna No. 3, Bulaksumur, Yogyakarta 55281, Indonesia*

[5]*Research Centre of Halal Products, Universitas Gadjah Mada, Jl. Kaliurang Km 4, Sekip, Yogyakarta 55281, Indonesia*

Abstract: *Analysis of non-halal components, such as pork and porcine gelatin, in food and pharmaceutical products is a need for halal authentication study. This research was aimed to develop a species-specific primer (SSP) to analyze DNA in porcine gelatin in soft candy using real-time PCR. The SSP to porcine DNA primer is designed using NCBI and Primer-BLAST software. The designed primer was subjected to a validation by assessing some parameters, including specificity, sensitivity, repeatability test, and linearity. The results showed that the real-time PCR with SSP targeting on mitochondrial D-loop specifically able to identify the presence of porcine DNA at an optimum annealing temperature of 50.5 °C. The coefficient of variation (CV) on repeatability analysis of Cq was 0.53%, and the efficiency value (E) for DNA amplification was 100%. Real-time PCR using D-LOOP porcine primer (forward: ACTTCATGGAACTCATGATCCG; reverse ATGTACGTTATGTCCCGTAACC) can also be successfully used for the identification of porcine gelatin DNA in soft candy.*

Keywords: *primer D-loop; porcine DNA; real-time PCR; halal authentication*

■ INTRODUCTION

All products, including pharmaceutical, food, personal care, chemical, biotechnology, and cosmetic products declared as halal and commercially available in Indonesia, must be halal certified, according to Indonesian Act No. 33 (2014). Consequently, the authentication analysis of products from the presence of non-halal components is highly required [1]. Along with increasing public awareness to consume halal food, the markets of halal products are believed to increase exponentially [2]. Analysis of non-halal components such as pork and porcine gelatin in food products presents a certain complexity to be carried out because these non-halal components are added in a matrix having a similar composition to halal components. For example, bovine gelatin was substituted with porcine gelatin in soft candy.

Various analytical methods have been developed for the analysis of porcine gelatin and pork, such as infrared spectroscopy combined with chemometrics [3], analytical method of lard extracted from pork in meatballs, and lard in meatball broth [4], liquid chromatography through amino acid analysis in gelatin [5], liquid chromatography-mass spectrometry for analysis of porcine gelatin and bovine gelatin [6], ELISA for analysis of porcine and bovine gelatin in food products [7]. However, these methods are lack of

selectivity. Therefore, DNA-based methods like real-time polymerase chain reaction have been developed.

The real-time PCR method is a method of choice for DNA analysis from certain species because DNA is found in almost all organisms and is not easily damaged even though it is subjected to food processing at high temperatures. DNA sequences are specific to certain types of organisms so that the PCR method can be used to detect a variety of non-halal components and can be applied to processed products [8]. Real-time PCR is capable of producing the amplification curve, which can be directly observed and analyzed quantitatively using fluorescent compounds such as SYBR Green. Real-time PCR using a species-specific primer (SSP) targeting the specific genes on mitochondrial displacement loop686 (D-loop) has been successful for the analysis of non-halal components such as pork in food products such as shredded and jerky [9]. Real-time PCR using SSP CYTBWB2-wb targeting on cytochrome-b has also been used for the detection of wild boar meat [10], pork [11], and dog meat [12] in meatballs. The SSP combined with real-time PCR has also been successfully used to detect porcine gelatin in shell capsules [13]. However, there is limited study on the detection of porcine gelatin in soft candy. In this study, the new designed SSP targeting on D-loop mitochondrial was developed for identification of porcine gelatin in soft candy

■ **EXPERIMENTAL SECTION**

Materials

The gelatin coming from bovine and porcine were obtained from Sigma (Aldrich, USA). Beef, pork, dog meat, goat meat, chicken meat, and rabbit meat were obtained from the traditional markets in Yogyakarta. The species-specific primers were designed and tested with software from the NCBI website and were subsequently ordered from Genetika Science Company (Jakarta, Indonesia). The other solvents and chemical reagents including sodium acetate, Tris-EDTA buffer, chloroform and *iso*-amyl alcohol were bought from E. Merck (Darmstadt, Germany). The market sample used in this study was soft candy sold in supermarkets in Indonesia.

Instrumentation

The primary tool used in this study is a set of real-time polymerase chain reaction (Biorad type CFX 96, USA).

Procedure

Primers design

The software of Primerquest from Integrated DNA Technologies was used to assist in designing the primers either Forward (F) or Reversed (R) specific to the DNA of wild boar. The DNA sequences of mitochondrial Cyt-b with an accession number of AF034253 was retrieved from GenBank (NCBI). BLAST was used to *in silico* test of designed primer. The selected F and R primers were:
Forward: ACTTCATGGAACTCATGATCCG;
Reverse: ATGTACGTTATGTCCCGTAACC
The melting temperatures of the F and R primers were 63 and 62 °C, respectively, and Guanine-Cytosine (GC) contents were 50% (F) and 45.5% (R) with amplicon product of 130 base pair (bp).

Preparation of soft candy

Preparation of candy samples was carried out as previously described [14]. Briefly, 20.0 g of gelatin were weighed and then soaked with 100 mL of water for 15 min. Next, 150 g of sugar and 5 mL of mango flavoring agent were dissolved in 100 mL of water. Gelatin that has been soaked was poured into a pan sugar and flavoring agent and then cooked while stirring. After thickening, the mixture was poured into a 10 × 20 cm baking pan and was left for 4 h until it solidified. After that, the candy was cut into pieces and sprinkled with sugar.

Isolation of DNA

The DNA was isolated from raw meats, porcine and bovine gelatin, and soft candy containing gelatins in its formulation. For isolation of DNA from raw meats, six types of meat, namely bovine (*Bos taurus*), Pigs (*Sus scrofa*), Goats (*Capra hircus*), chickens (*Gallus gallus*), rabbits (*Lepus curpaeums*), and dogs (*Canis lupus familiaris*) were used. This isolated DNA from raw meats was used for specificity testing of the designed primer. The crushed meat (approximately 200 mg) was weighed, added with 700 µL of buffer lysis, heated for

15 min at 65 °C, then added with 10 μL of proteinase K (2%), and homogenized using a vortex. The mixture was then incubated at a water temperature of 65 °C for 55 min, vortexed every 10 min, and then incubated in the water bath at a temperature of 38 °C for 30 min, and followed by centrifuging the supernatant at 13,000 rpm for 15 min. The supernatant was placed into a new Eppendorf tube, added with 0.5× volume of phenol and 0.5× volume of chloroform-*iso*-amyl alcohol. The mixture was shaken with a shaker for 30 min, followed by a centrifugation process at 13,000 rpm for 15 min. The supernatant was then removed, followed by addition of 1× volume of chloroform, homogenized, and subsequently centrifuged at 13,000 rpm for 15 min. The supernatant was moved and followed by the addition of 0.1× volume of 3 M sodium acetate pH 5.2 and 2× volume of absolute ethanol, and then incubated at -80 °C overnight. The mixture was centrifuged (at 4 °C) for 5 min at 13,000 rpm, and the supernatant was immediately discarded. The remaining ethanol was dried in laminar airflow for approximately 10 min, and each sample was followed by the addition of 40 μL of Tris-EDTA buffer and stored in the refrigerator -20 °C for subsequent analysis [15].

For isolation of gelatin and candy, samples containing gelatin were prepared by weighing approximately 100 mg (standard gelatin) or 200 mg of reference candies. Then, the gelatin and candy samples were incubated at a water temperature of 65 °C until dissolved with vortex every 15 min. Then, the mixture was added with 1 mL of absolute ethanol and followed by a centrifugation process at 13,000 rpm for 3 min at room temperature. The supernatant is then removed, and the obtained precipitate was subjected to the addition of 800 μL of heated buffer lysis and 20 μL of proteinase K (2%) and shaken manually until homogeneous. The mixture was incubated in a water bath at 65 °C for 75 min, vortexed every 15 min. Each sample was then added with 0.5x volume of phenol and 0.5× volume of chloroform-*iso*-amyl alcohol and shaken for 40 min. Then, the mixture was centrifuged for 30 min at 14,500 rpm at room temperature. The supernatant was subjected to the addition of 1× volume of chloroform and shaken for 15 min. The mixture was subsequently subjected to

centrifugation process at 14000 rpm for 10 min. Furthermore, the supernatant was subjected to the addition of 3× volume of Na-acetate 3 M, 2× volume of ethanol, and incubated at -80 °C overnight, followed by centrifugation at 14,500 rpm for 5 min at 4 °C. Finally, the supernatant was immediately discarded, and the obtained DNAs were used for real-time PCR analysis.

Determination of purity and concentration of isolated DNA

The purity index of DNAs was analyzed using an ultraviolet spectrophotometer at a wavelength λ of 260 and 280 nm. The levels of analytes (concentration) of DNA were calculated based on the absorbance values at λ of 260 nm multiplied by 50 μg/mL and the dilution factor.

Real-time PCR analysis

The annealing temperature of the primer was optimized by applying the temperature range of 46–52 °C based on the prediction of the melting temperature of the primer. Some temperatures were subjected to optimization, at 49.5, 50.5, and 59.5 °C.

The real-time PCR analysis was performed in reaction tube (total volume of 20 μL) consisting of 10 μL of SYBR Green® universal PCR master mix, 1 μL of forward and reverse primer (10μm/μL), 2 μL of template DNA (50 ng) and 6 μL of free nuclease water. The temperature program used was 95 °C for 30 sec followed by 40 cycles at 95 °C for 5 sec for the denaturation stage. The next stage was the annealing of primer at 50.5 °C that has been previously optimized for 30 sec and 72 °C for 10 sec for the extension (elongation) stage. The melting curve of the PCR reaction was performed at 65–95 °C with a slope of 0.5 °C/5 sec.

Validation of the real-time PCR method

The real time-PCR using designed primer was validated to meet its purpose according to Codex Alimentarius Commission [16]. Some parameters, namely specificity, amplification efficiency, the limit of detection, and repeatability, were evaluated. The primer specificity was confirmed by amplifying DNAs extracted from raw meats and negative control called no template control (NTC). The standard curves for linearity tests

were made based on the amplification of reference candies with various concentrations. Linear regression equation made by comparing the value of Quantification circle (Cq) in *y-axis* to the log value of DNA concentration in *x-axis* was used to determine the efficiency value (E) and limit of detection. The repeatability of the real-time PCR method was assessed based on the coefficient of variation (CV) Cq values during amplification.

Analysis of commercial samples

The validated real time-PCR using species-specific primer was then applied for analysis of porcine gelatin in various candy products commercially available in markets around Yogyakarta, Indonesia.

Data analysis

The data analysis for real-time PCR was done with a standard curve created by comparing the value of Quantification circle (Cq) against the log value of DNA concentration. Efficiency (E) real-time PCR is calculated according to Eq. 1 [17]:

$$\%E = \left(10^{-1/\text{slope}} - 1\right) \times 100\% \tag{1}$$

■ RESULTS AND DISCUSSION

The primary design was carried out using the Primary Quest Tool online software with access code AF034253 targeting on mitochondrial D-loop of *Sus scrofa* (Table 1). The primers were selected by considering several primary design parameters, such as 15–30 bp in length, 40–60% GC content, G and C nucleotides evenly distributed, and the primer and melting temperature (Tm) range at 50–60 °C [18]. The Tm in primers was used to determine the optimization of the annealing temperature of target DNA. Too high or too low of Tm causes the target DNA amplicon to not specifically attach. The GC content in a pair of primers is almost the same. Inadequate GC content can cause the presence of polypurine and polypyrimidine, which will affect the primer not specifically attached [19].

The purity of isolated DNA from fresh meat showed high purity with A260/A280 ratio values obtained close to 1.8. The results of DNA purity from the standard pork gelatin and reference candy samples have ratio values below 1.8. The DNA isolates have experienced degradation during gelatin production from the original material. The DNA purity results of the gelatin are shown in Table 2. The designed primers were then used to optimize the temperature of the porcine DNA attachment to DNA isolates from other meats (beef, pig, chicken, goat, dog, and rabbit).

The primer optimization was carried out on meat DNA isolates because they were easier to obtain variations of animal species likely used as a basis for gelatin than gelatin variations from different species sources. The results of primer optimization can be seen in Fig. 1(a). The observations on the melt peak curve indicated one peak produced at a temperature of 78.0 °C, shown in Fig. 1(b). The annealing temperature optimization is based on several parameters, namely the

Table 1. Results of primary and reverse forward primary designs

Sequence (5'-3')	Nucleotide bases (bp)	Tm (°C)	GC (%)
F: ACTTCATGGAACTCATGATCCG	22	58.20	45.45
R: ATGTACGTTATGTCCCGTAACC	22	57.95	45.45

Table 2. Quantitative results of gelatin DNA and DNA candy references with a nanodrop

No.	Sample Type	A_{260}	A_{280}	Concentration (ng/μL)	Purity index
1	Porcine Gelatin	2.8837	1.3757	1883.74	1.37
2	Bovine Gelatin	2.6668	1.1788	1666.81	1.41
3	Soft candy reference	1.1177	0.8502	1117.74	1.31
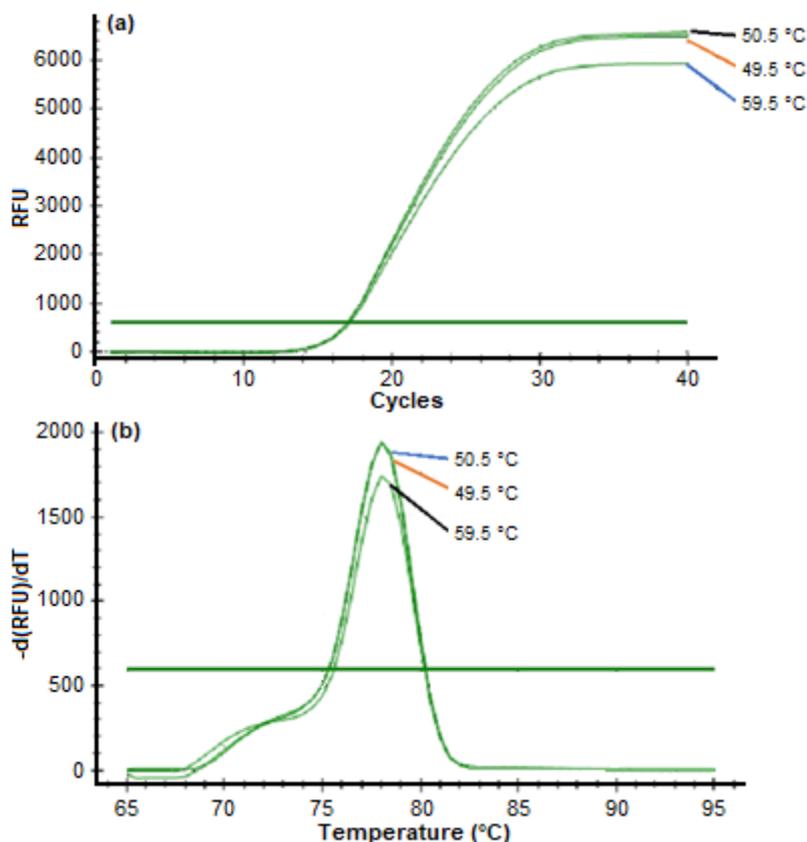
Fig 1. The results of the optimization of D-loop primary attachment temperature in 40-cycle pig DNA, using real-time PCR, (a) amplification, and (b) melting curve
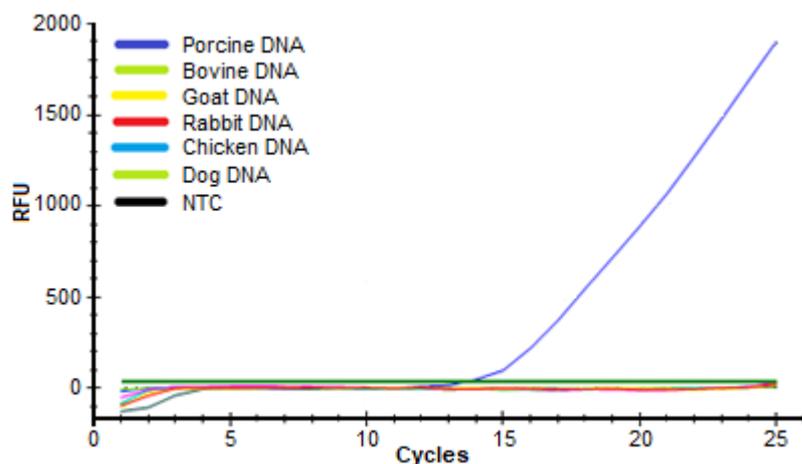
Fig 2. Amplification of the results of the porcine primary specifications against the DNA of other animals

lowest cycle quantification (Cq) value, the minimum non-specific product, and the minimum dimer primary products [20-21]. The optimization of the primary annealing temperature of porcine DNA in this study was 50.5 °C, with a value of Cq of 17.16. Furthermore, the specificity of the primer was tested at the optimum annealing temperature. Based on Fig. 2, it can be seen that the designed primer is specific, in which porcine DNA is amplified while other DNAs along with the negative template control (NTC) are not amplified.

The specific primer was subjected to validation by performing several performance characteristics, including linearity and repeatability tests using the reference DNA samples made from porcine gelatin. Fig. 3 shows a linear regression curve with an R^2 value of 0.971 and an efficiency value of 100.0%. The obtained efficiency values meet the acceptance requirements in the range of 90–110%, but the R^2 value obtained does not meet the qPCR method qualitative and quantitative testing criteria, namely $R^2 = 0.980$ [17]. The lower R^2 values obtained than the required acceptance criteria are probably due to the inhomogeneity of the reference candy products.

The repeatability test to measure the precision of the amplification results from the reference DNA samples containing porcine gelatin was performed by calculating the standard deviation (SD) and the coefficient of variation (CV). The repeatability expresses the closeness of results between a series of measurements obtained from homogeneous samples under predetermined conditions in a short time [22]. The amplification data obtained during precision studies can be seen in Fig. 4. The DNA repeatability test of the reference candy containing pigs resulted in CV = 0.53% (Table 3), which is acceptable for q-PCR, namely CV ≤ 25% [19].
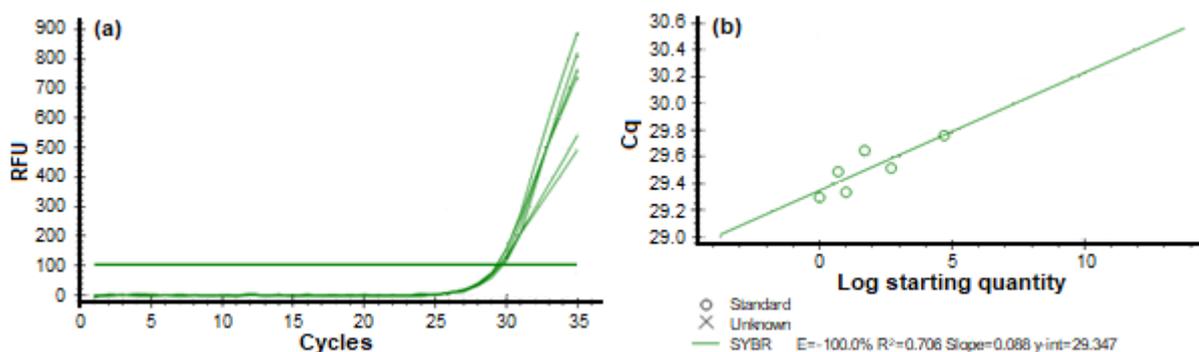
Fig 3. The reference of candy DNA dilution series made from porcine gelatin, (a) amplification and (b) standard curve
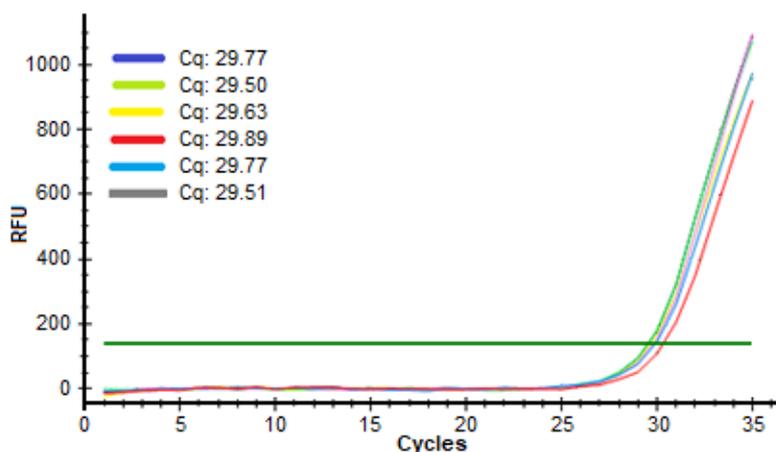
Fig 4. DNA test results of reference candy stability containing porcine gelatin

Table 3. DNA test results for reference candy containing pig gelatin

Sample	Cq	average Cq	SD	CV (%)
Porcine DNA gelatin concentration 50 (ng/µL)	29.77	29.68	0.1575	0.53
	29.50			
	29.63			
	29.89			
	29.77			
	29.51			
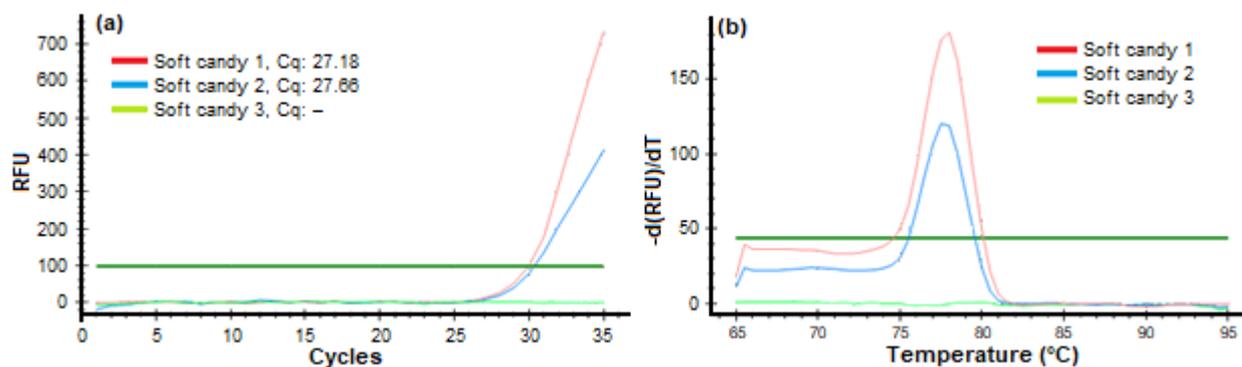
Fig 5. The results of DNA isolates from candy market samples, (a) amplification and (b) melting curve analysis

Therefore, this method is quite precise and precise for analysis candy containing pork gelatin.

The validated method was then applied to commercial samples, and the results can be seen in Fig. 5. There are three market samples tested with third specifications that do not have a halal label, and it turns out that two samples were amplified using primers that have been validated, which means that the sample is likely to contain pig DNA. The results of the melt curve show that both samples have the same Tm at 78 °C. Meanwhile, one sample was not amplified with the designed primer, so that it is most likely that the sample does not contain pig DNA.

■ CONCLUSION

The real-time PCR method with the D-loop primer (forward: ACTTCATGGAACTCATGATCCG; reverse ATGTACGTTATGTCCCGTAACC) can be applied to detect porcine gelatin in soft candy. The optimum annealing temperature was 50.5 °C. The coefficient of variation (CV) on repeatability analysis of Cq was 0.53%, and the efficiency value (E) for DNA amplification was 100%. The developed method is successfully used for the analysis of actual samples with acceptable precision and accuracy. This method can be used as a standard method for the detection of non-halal components (porcine gelatin) for halal authentication study.

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■ AUTHOR CONTRIBUTIONS

Nina Salamah and Yuny Erwanto conducted the experiment, Sudibyo Martono and Abdul Rohman conducted the data analysis calculations, Nina Salamah, Yuny Erwanto, Sudibyo Martono, and Abdul Rohman wrote and revised the manuscript. All authors agreed to the final version of this manuscript.

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