Short Communication:

The Use of Real-Time Polymerase Chain Reaction Combined with Specific-Species Primer for Analysis of Dog Meat DNA in Meatball

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Abstract: *The presence of dog meat is a crucial issue because dog meat is non-halal meat for Muslims. The objective of this study was to design and validate species-specific primer for the identification of dog meat DNA in meatball using real-time polymerase chain reaction (real-time PCR). The specific primer targeting mitochondrial cytochrome c oxidase subunit 1 (CO1) was validated. The specific primers used were designed using Integrated DNA Technologies (IDT) software and subjected to NCBI BLAST procedure. The candidate primers were tested for specificity study using several DNAs from fresh meat of pork, chicken, beef, lamb, and rat. The method was also validated by determining several parameters of linearity, sensitivity, precision, and efficiency. The results showed that primer could amplify specifically DNA target at an optimized annealing temperature of 56.6 °C. The limit of detection (LoD) obtained was 5 ng DNA, corresponding to 2.5% of dog meat in a meatball. The repeatability evaluation, expressed with relative standard deviation (RSD), and efficiency value was in the acceptable range (RSD < 25% and efficiency (90–105%). This method was successfully used for the analysis of marketed samples. Real-time PCR can be used as a standard method in halal authentication analysis through DNA analysis.*

Keywords: *dog meat; meatball; real-time polymerase chain reaction; halal authentication*

■ INTRODUCTION

Currently, the trade of meat and meat products such as meatball has occurred globally and possesses a high commercial value. Due to the high consumption of meat products and the high value of meat trade, some unethical economic players made efforts to adulterate meat products to gain economic profits [1-2]. For Indonesian society, beef meatball is one of the favorite foods due to its taste and protein source. It is available in traditional and modern markets [3-4]. However, due to the difference in price among meat, some meatball producers mixed or substituted beef with lower-priced meat. One of the potential meats used for beef adulteration is dog meat. In certain countries, like Indonesia and Malaysia, the dog is available at lower prices than beef meat. Besides, some communities in certain countries like China, Myanmar, South Korea, and Vietnam consume dog meats [5].

Dog meat is non-halal to be consumed, and the followers of Islamic and Buddhism religions are restricted from consuming any food containing dog meat [6-7]. Indonesian Regulation Act No. 33 (2014) stipulated that any halal products (food, cosmetics, personal care, pharmaceuticals) spreading in Indonesia must be certified. Indonesian newspaper has reported the contamination of beef meatball with dog meat. Consequently, to implement this act and meet the requirements of halal product authentication, some

scientists have developed instrumental analytical techniques that can detect non-halal components present in any products, including meatballs [8]. In the future, the developed methods should be standardized and are available among Islamic countries.

Numerous methods have been used for identification of non-halal meats in food products, including differential scanning calorimetry for thermal characterization of pork [9], analysis pork using fast gas chromatography-detector of surface acoustic wave for analysis of volatile compounds specific for pork fat [10], gas chromatography in combination with TOF-MS (time of flight mass spectrometer) and high-performance liquid chromatography using several detectors such as refractive index and mass spectrometer for analysis of fatty acid composition and triglyceride, respectively [11-12], spectroscopic techniques of infrared and nuclear magnetic resonance [13-14], and DNA-based techniques using polymerase chain reaction [15].

Real time-polymerase chain reaction (real-time PCR) is a method of choice for identifying species origin in food products [16]. It is taken into account as a standard method for the analysis of non-halal meat [17]. Real-time PCR combined with fluorescence molecules was widely used for monitoring the amplification products obtained in each PCR cycle [18]. Compared to conventional PCR, real-time PCR offers excellent sensitivity, high specificity, broader dynamic detection range, reproducible results, low contamination risk, and reduced hand-on time. In addition, real-time PCR does not need post-PCR analysis, such as electrophoresis [19-20]. In this respect, currently, real-time PCR appears to be the most effective and reliable method to identify and quantify trace amounts of different animal origin in the complex food samples [21].

Real-time PCR using species-specific primer has been used for analysis of non-halal meat, especially pork in food products [22], wild boar meat in meatball products [23], donkey meat and pork in raw and heat-processed meats [21], and rat's meat DNA in meatball products [24]. Real-time PCR using some primers targeting on specific genes was also reported for the identification of dog DNA. Rahman et al. [4] have used primers targeting the cytochrome b gene (Cyt-b) in meatball samples. Primers targeting on Cyt-b gene were used for the analysis of DNA dog meat in commercial frankfurters [25] and in meatball samples [26]. However, the application of PCR using primers targeting mitochondrial cytochrome c oxidase subunit 1 (COI) was limited. The objective of this research was to design species-specific primers targeting on COI gene and validate real-time PCR using that primer for qualitative and quantitative analyses of dog meat DNA in meatball products.

■ EXPERIMENTAL SECTION

Materials

Dog meat was obtained from three different slaughters around Purwokerto, central Java, and Yogyakarta, Indonesia. Beef, chicken, lamb, spices, common food preservatives, as well as other food additives, were purchased from local and supermarkets around Yogyakarta, Indonesia. The commercial meatball samples were bought from several markets around Purwokerto and Yogyakarta. All solvents and chemicals used were of pro-analytical grade. The positive control used was DNA extracted from dog meat, while the negative controls were DNA extracted from cow, chicken, goat, pork, and rat. Meatballs were prepared and formulated as in Purnomo and Rahardiyan [3] by emulsifying 90% finely ground meats with 10% oats. The mixture was then mixed with other components, namely saline 0.01% (wt/wt) and spices, shaped as ping pong ball size, and finally subjected to boiling water for 15 min.

Procedure

Primer design

The species-specific primers were designed using the IDT (Integrated DNA Technologies) with Gen Bank number EU408308.1. The target primer used is mitochondrial COI-1 98470455 (forward)-98470454 (reverse), i.e., forward: 5'-CCT CAA CAT TCC CTA GGT TTAT-3'; reverse: 5'-CCT ATA GAG GAG ACG GTA TTT-3'. The designed primer was subjected to NCBI BLAST analysis in order to confirm its specificity to dog meat DNA *in silico* [24].

Real-time PCR analysis

The DNA isolation was done using Favorgen DNA isolation kit® according to the manufacturer's protocol. Amplification was performed in a final volume of 20 µL containing 30 ng isolated DNA, 10 µL Ssofast Evagreen® supermix (Biorad, USA), 1 µL (10 mmol) of each primer, and 6 µL of free nuclease water. The amplification was performed with a real-time PCR using PCR CFX96® (Biorad, USA). The thermal cycler protocol was as follows: pre-denaturation at 94 °C for 4 min, followed by 25 cycles of denaturation at 94 °C for 30 sec, annealing at 56.6 °C for 30 sec, and extension at 72 °C for 30 sec [27].

Validation of real-time PCR using designed specifically primer

The primer designed was further validated by determining several parameters, which include specificity, efficiency, sensitivity, and repeatability, according to Perestam et al. [28] and Safdar et al. [29]. The specificity test of primer COI-1 was evaluated by amplifying DNA extracted from several types of meat (cow, chicken, goat, and rat), and DNA extracted from positive control (meatball containing 100% dog meat) and negative control (meatball containing 100% beef). The sensitivity of real-time PCR using primer COI was expressed by the limit of detection (LoD). The evaluation of LoD was carried out by making dilution series of DNA extracted from dog meat at a concentration of 1000, 500, 200, 150, 100, 10, and 1 pg of DNA 100% dog meat. LoD value was the lowest amount of DNA that could be amplified with reproducible cycle threshold (Ct) value. The repeatability assay for precision evaluation was performed by replicating these dilution series in three replicates [30].

Application of real time-PCR for analysis of commercial samples

The commercial meatball samples obtained from several markets in Purwokerto and Yogyakarta, Indonesia, were analyzed by validated real-time PCR, using COI-1 primer and set RT-PCR at the optimum annealing temperature.

■ RESULTS AND DISCUSSION

To develop a robust and reliable method for analysis of dog meat DNA in food products, a specific primer combined with real-time PCR is essential [29]. In this study, the primer targeting the mitochondrial cytochrome oxidase I gene (COI) was designed using the IDT software and used to identify and quantify dog meat DNA using real-time PCR. This gene was chosen because it revealed an appropriate degree variability of intra- and inter-species. Besides, this gene also offers a high number of copies per cell, which increases the sensitivity of real-time assay significantly and contributes to the survival of few DNA copies when tissue has been subjected to extreme processing conditions, such as sterilization and boiling processes [30].

In processed food such as meatball, DNA may be degraded into short fragments that make it difficult during PCR amplification. The important thing for successful PCR analysis is to obtain sufficient DNA templates. Therefore, in this study, DNA was extracted using a commercial kit to get adequate quantities. The isolated DNAs from different animal tissues were analyzed using Nanodrop at 260 and 280 nm, which were useful to find out the concentration and purity of DNA isolates. The purity of DNAs was in the range around 1.8–2.0, indicating that DNA was pure enough to be used as a DNA template for PCR amplification. During PCR analysis, the annealing temperature, associated with a primary attachment of primers on the target DNA, is an important factor in the process of real-time PCR amplification. A range of annealing temperature (45.3–57.3 °C) was examined, and the optimal temperature for attachment of primer was 56.6 °C. At this temperature, the DNA template (dog DNA) could be amplified maximally with the lowest value of the Cq (quantification cycle) and the highest RFU (ratio of fluorescence unit) values, as shown in Fig. 1. There were no dimer primers and non-specific products observed during PCR amplification, which indicated that optimum condition was reached.

The designed COI-1 primer was validated to examine the capability of primer to amplify the DNA target. The validity evaluation included the specificity, sensitivity expressed by a limit of detection, efficiency, and repeatability. The specificity test was carried out by amplifying DNAs extracted from different animal tissues

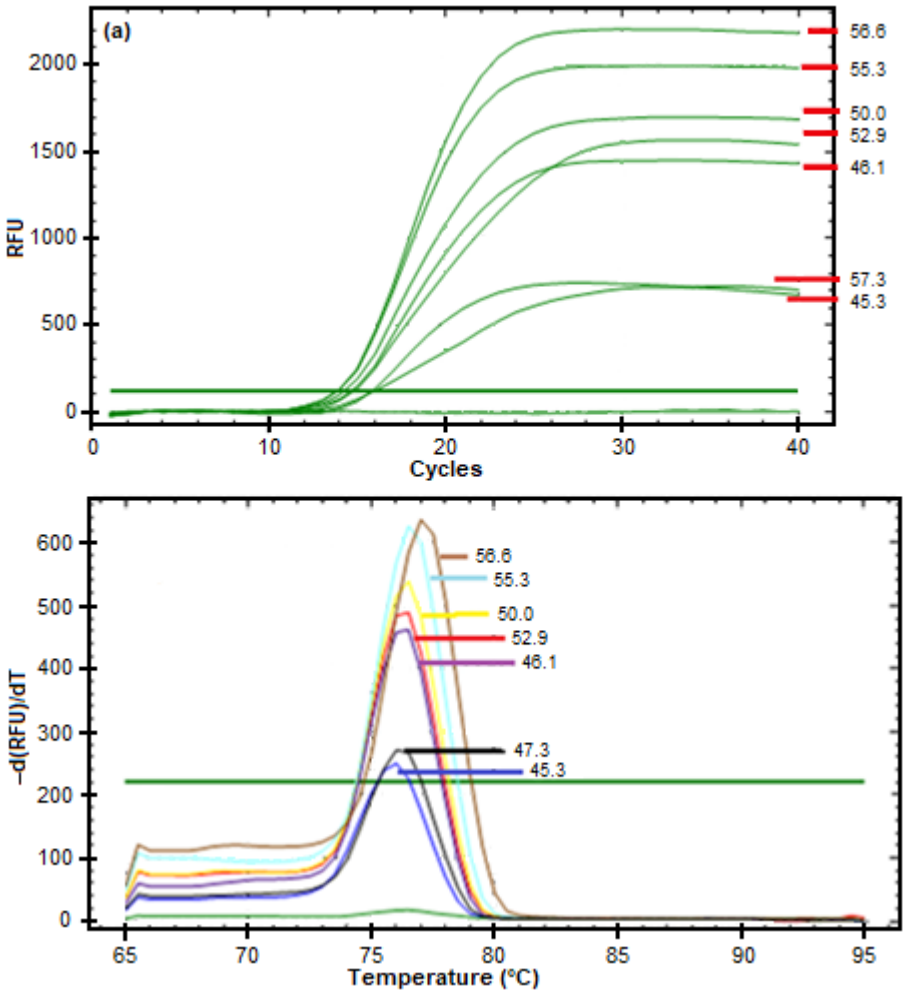

Fig 1. The Amplification curves (a) and melting peak (b) during the optimization of annealing temperature of COI primers to amplify DNA target extracted from dog meat. The optimum annealing temperature was 56.6 °C

(cow, chicken, goat, pork, and rat). Fig. 2 exhibited the amplification results using primer COI in which the primer specifically amplify DNA from dog meat. In contrast, other DNAs were not amplified until 30 cycles because there is no amplification (Fig. 2). This amplification proved that the designed primer was specific for dog DNA. For determination of limit of detection (LoD), the serial dilutions of dog DNA extracted at concentrations of 1000, 500, 200, 150, 100, 10, 5, and 1 pg were prepared. The COI-1 primer could amplify 5 pg, while at 1 pg, dog meat DNA is not amplified to 30 cycles (Fig. 3). Therefore, it could be stated that the LoD value of real-time PCR for analysis of dog DNA was 5 pg. Manalu et al. [26] reported that Real-PCR using primers targeting on Cyt-b gene primer could detect the presence of DNA at a concentration as low as 0.25 ng/mL, corresponding to 1% of dog meat in beef meatballs. Rahman et al. [4] reported LoD values of 0.02 ng dog DNA corresponding to 0.1% of dog meat in meatball samples, and this LoD value was similar to that reported by Ali et al. [25] in frankfurters using chicken and beef meat. From this result, primers targeting Cyt-b were more sensitive than those targeting on COI gene.

For efficiency evaluation, a calibration curve relating between log concentration of DNA (*x-axis*) and Ct value (*y-axis*) was prepared. Fig. 4(a) showed linear regression for such correlation, which resulted in a coefficient of determination (R^2) value of 0.984, with intercept and slope values of 27.799 and -3.448, respectively, using DNA extracted from the mixture of

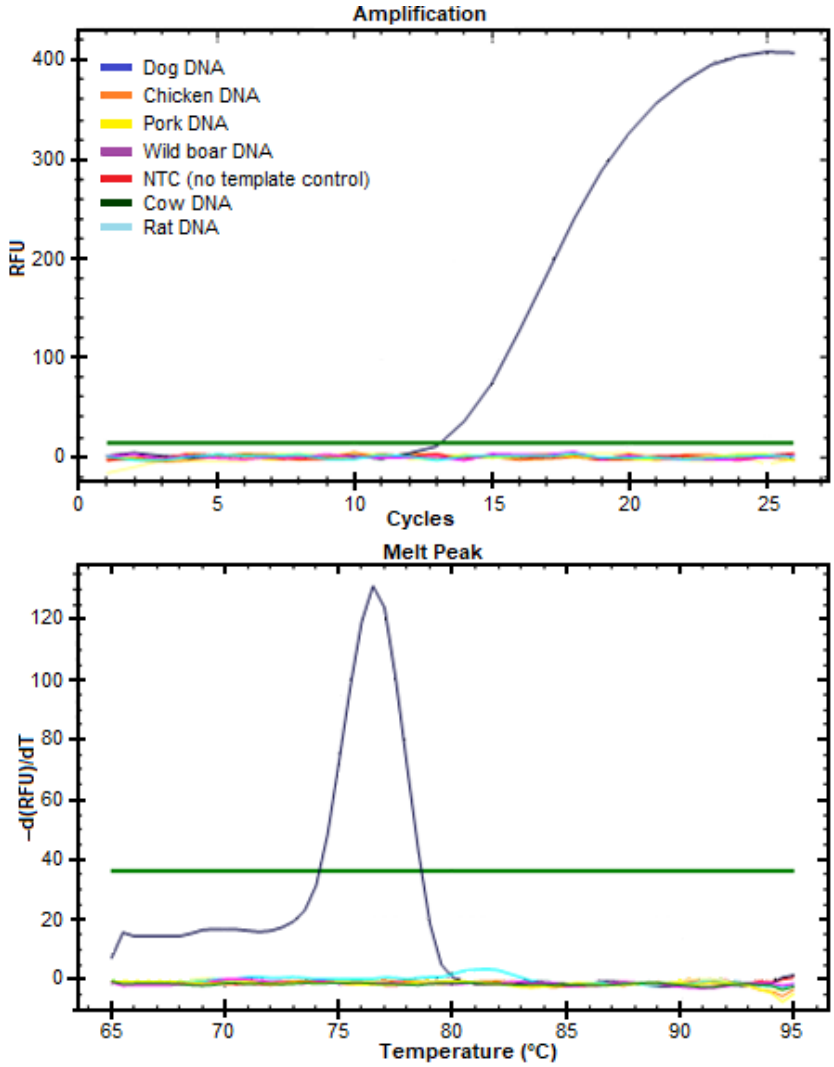

Fig 2. The specificity test of primers on dog meat DNA from compared with that on DNAs extracted from cow, chicken, pork, goat, and rat

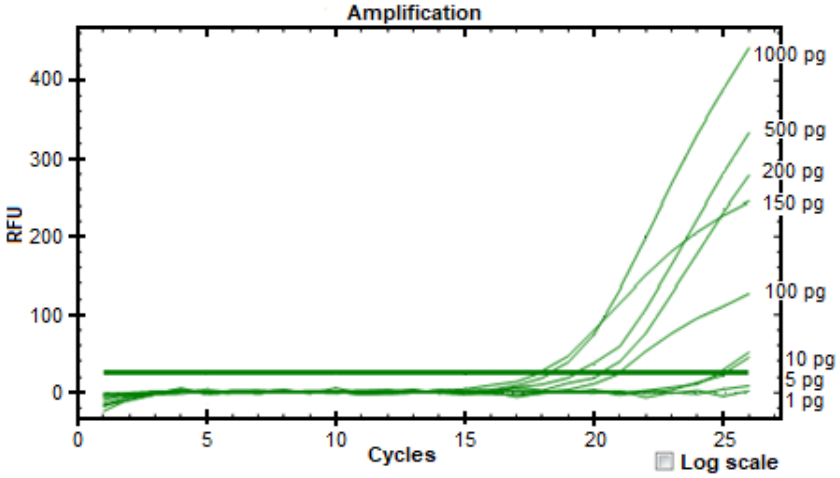

Fig 3. The amplification curve of dog DNA at concentrations of 1000, 500, 200, 150, 100, 10, 5, and 1 pg for determination of limit of detection

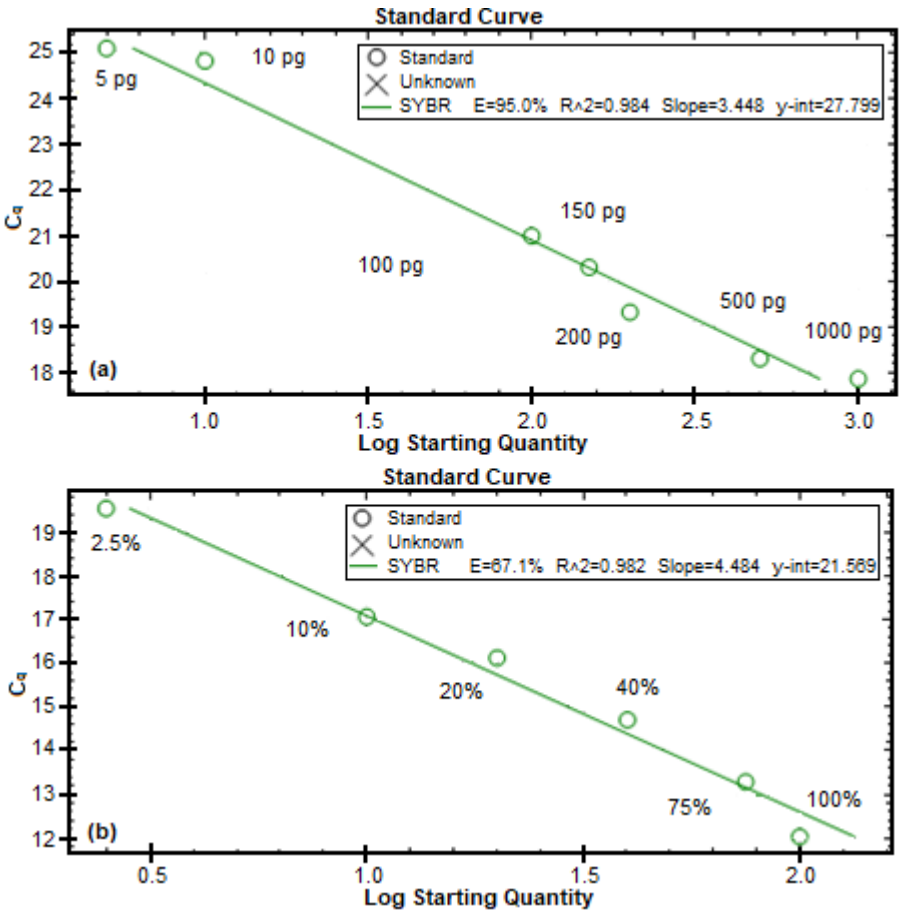

Fig 4. The standard curve for the relationship between log DNA concentration extracted from dog meat-beef at 1000, 500, 200, 150, 100, 10, 5, and 1 pg (*x-axis*) and Ct values (*y-axis*), showing R^2 of 0.984 and E value of 95% (a), as well as in mixed meatball of dog meat-beef with the concentration of 2.5, 10, 20, 40, 75, and 100% showing R^2 value of 0.982 and E value of 67.1%

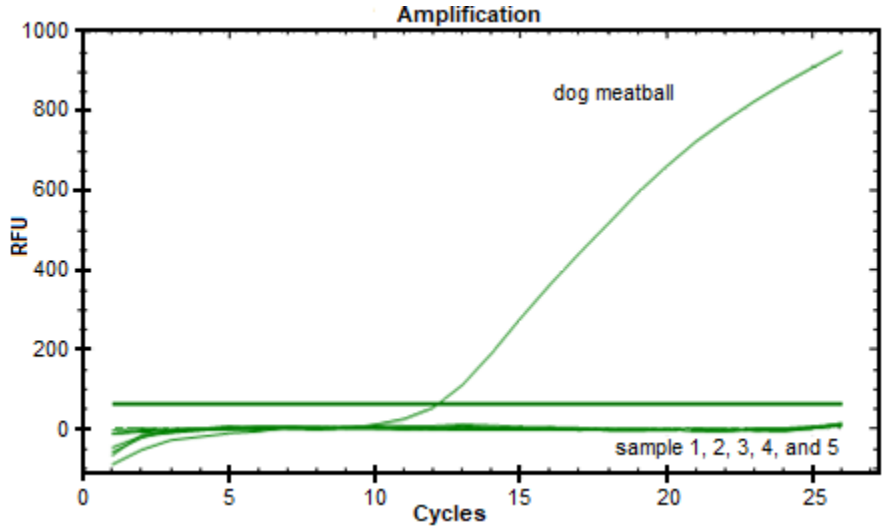

Fig 5. The amplification profile of DNA extracted from commercial meatballs showed no amplification peak and positive control having amplification peak at Ct of 10

dog meat-beef. This indicated that 98.4% variation of Ct value was due to the variation in DNA concentration. The P-value obtained from the ANOVA test was < 0.01, meaning that the significant correlation existed between log concentration of DNA and Ct value. The efficiency value (E) obtained was 95%, which met the requirement of acceptable E values, i.e., in the range of 90–105% [31]. Using DNA extracted from meatball containing different concentrations of beef and dog meat, R^2, slope, and intercept values obtained were 0.982, -4.484, and 21.569, respectively, as in Fig. 4(b). The E value obtained was 67.1%.

The precision of real-time PCR was evaluated by the repeatability test. Template DNA at a concentration of 100 ng was amplified using COI primer with six replicates, and a relative standard deviation (RSD) value of 11.46% was obtained. Real-time PCR analysis was considered precise if the RSD value < 25%. The validated real-time PCR method was subsequently used for the analysis of marketed samples. From the amplification profile (Fig. 5), all evaluated samples were not amplified, meaning that all samples were not detected to contain dog DNA. Real-time PCR using specific primer targeting on mitochondria COI-1 can be used for identification of dog meat in food samples, and further can be standardized on halal food analysis.

■ CONCLUSION

Real-time PCR using a specific primer of mitochondrial COI-1 at an annealing temperature of 56.6 °C was successfully used to identify and to quantify dog meat in a meatball. The validation results revealed that real-time PCR was fit for purposes for the identification of dog meat DNA.

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■ AUTHOR CONTRIBUTION

SRW and AR designed and conducted research activities and prepared manuscript. SM and Sudjadi designed research, analyzed data, and drafted manuscript.

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