Short Communication

The use of species-specific primer targeting on D-loop mitochondrial for identification of wild boar meat in meatball formulation

Rien Larasati Arini[1], Dwiky Ramadhani[1], Ni wayan Pebriyanti[1], Sismindari [2] and Abdul Rohman [2,#]

AFFILIATIONS

[1]*Faculty of Pharmacy, Gadjah Mada University, Sekip Utara, Yogyakarta 55281, Indonesia.*

[2]*Departement of Pharmaceutical Chemistry, Gadjah Mada University, Sekip Utara, Yogyakarta 55281, Indonesia.*

ABSTRACT

Objective: The study was intended to design the new specific primer targeting on mitochondrial D-Loop gene (D-Loop 443 primer) combined with a real-time polymerase chain reaction (PCR) for the analysis of wild boar meat (WBM) in food products of meatball.

Materials and methods: The primer was designed and subjected to primer-basic local alignment search tool using National Center for Biotechnology Information software. Validation of real-time PCR using designed primer was performed by evaluation of several performance characteristics which included specificity, sensitivity, repeatability, linearity, and efficiency.

Results: The results showed that the D-loop primer could be attached at 60.7°C and no amplification was detected against other species confirming the specificity of the primers. The limits of detection were found to be 4.68 ng and 2.34 ng using DNA extracted from WBM and that extracted from wild boar in meatball product. The D-Loop 443 primer was successfully used for the analysis of commercial meatball samples.

Conclusion: The developed method can be proposed as a standard method for the identification of WBM in meatball to support halal products authentication.

CORRESPONDENCE:

 # Abdul Rohman,
Departement of Pharmaceutical Chemistry, Gadjah Mada University, Sekip Utara, Yogyakarta 55281, Indonesia.
E-mail: abdul_kimfar@gmail.com

How to cite: Arini RL, Ramadhani D, Pebriyanti NW, Sismindari, Rohman A. The use of species-specific primer targeting on D-loop mitochondrial for identification of wild boar meat in meatball formulation. Journal of Advanced Veterinary and Animal Research. 2018; 5(3):361-368.

http://bdvets.org/javar/

Arini et al./ *J. Adv. Vet. Anim. Res., 5(3):361-368, September 2018* **361**

INTRODUCTION

Meat adulteration such as substitution of beef with non-halal meat such as pork is a worldwide problem, especially in Muslim countries. Indonesia is one of the largest Muslim community which concerns halal food products as stipulated in Indonesian Act No. 33, 2014 related to Halal Product Assurance. Due to the difference in price, unethical seller tried to substitute beef with lower price meats such as pork and wild boar meat to get economical profits (Montowska and Pospiech, 2011). Wild boar meat (WBM) is one of the meat types which Muslim communities are forbidden to consume it (Nakyinsige et al., 2012).

Among the meat-based food products that are commonly adulterated is a meatball, especially which is prepared from beef. Meatball is considered as meat-based food products consumed by Indonesian and other countries for the reason that meat contained in meatball can be a good protein source needed by the human body (Purnomo and Rahardiyan, 2008). As a consequence, reliable techniques based on physico-chemical and biological methods capable of detecting WBM as an adulterant in beef meat must be developed. Some methods used for the authentication of meat-based products were fluorescence spectroscopy (Sahar et al., 2016), near spectroscopy (Alamprese et al., 2013; Kamruzzaman et al., 2013; Dalle Zotte et al., 2014), Fourier transform infrared spectroscopy (Rohman et al., 2011; Guntarty et al., 2015), differential scanning calorimetry (Guntarti et al., 2017), electronic nose in combination with mass spectrometer (Nurjuliana et al., 2011), and chromatography-based methods such as gas chromatography and high-performance liquid chromatography (Dugo et al., 2006; Indrasti et al., 2010). However, these methods have the main drawbacks, namely, they are not specific enough and are only applicable to certain types of matrix samples. To overcome these problems, more specific methods based on biological markers of DNA and protein have been developed.

Protein-based methods such as enzyme-linked immunosorbent assays (ELISA) and immunoassays are widely used for meat identification (Hsieh and Ofori, 2014). These methods may also be commercially available in the form of test kits, having characteristics of user-friendly, suitable for routine monitoring due to its simplicity but have a limitation to be used during the analysis of food products treated with extensive heat processing. Protein-based methods also required extensive and sophisticated instruments and high skill analyst (Meira et al., 2017). As a consequence, method-based DNA such as polymerase chain reaction (PCR) is an emerging analytical technique for meat identification.

PCR used for authentication analysis of meat was based on detection of specific DNA for certain species. DNA is also stable at high temperatures, therefore, DNA can be analyzed either in fresh and frozen food products or in processed and degraded food products. Besides, DNA is also present in all organisms (Hopwood et al., 1999; Murugaiah et al., 2009). The development of PCR technology in the form of real-time PCR allows specifically the quantitative analysis of DNA in various samples, offering high sensitivity with a low value of detection limits (Navarro et al., 2015). Real-time PCR is also capable of analyzing analytes with reproducible results in a short time. Real-time PCR with species-specific primer has been used for the identification of non-halal meats, namely pork in raw meat (Yusop et al., 2012), dendeng (Maryam et al., 2016), abon (Rahmawati et al., 2016), rat meat (Widyasari et al., 2015), as well as cat, dog, and monkey meats (Ali et al., 2005). In the present study, we have designed species-specific targeting on displacement loop (D-loop) for the identification of WBM in a beef meatball. The designed primer combined real-time PCR was also validated and applied for the analysis of commercial meatball.

MATERIALS AND METHODS

Samples: WBM was obtained from Palembang, South Sumatera, Indonesia. Beef, chicken, pork, and goat meat were purchased from a local retailer around Yogyakarta, Indonesia. Dog meat was obtained from slaughterhouses in Purwokerto, Indonesia. Monkey meat was kindly obtained from Animal Section of Integrated Laboratory of Research and Testing Gadjah Mada University (LPPT-UGM), Yogyakarta, Indonesia. The meatball samples that are commercially available were acquired from 15 different local markets randomly taken from Yogyakarta Province, Indonesia.

Primers design: A pair of a wild boar-specific primer were designed using *online software* from Integrated DNA Technologies with the URL address of https://sg.idtdna.com/Primerquest/Home/Index. DNA sequences of mitochondrial D-loop (Accession No. KM016443) were retrieved from GenBank NCBI (National Center for Biotechnology Information) at the URL address of https://www.ncbi.nlm.nih.gov. BLAST (primer-basic local alignment search tool) was used to check primer specificity *in silico* using tools at http://blast.ncbi.nlm.

nih.gov/Blast.cgi which allowed identification of similar local regions among homolog sequences in different species and to evaluate the significant matches statistically. The oligonucleotides of designed primers used in this study were: D-loop 443F: 5´-ACT AAT CAG CCC ATG CTC AC-3´ and D-loop 443R: 5´- TGA CTG TGT TAG GGC CTT TG-3´. The primers were synthesized by Integrated DNA technologies Pte. Ltd (Singapore, Republic of Singapore).

Preparation of reference meatballs: The reference meatballs were prepared using binary mixtures of 0–100, 1–99, 5–95, 10–90, 25–75, 50–50, 75–25 and 100%–0% (wt/wt) of WBM–beef. After the addition of tapioca flour, each mixture was homogenized using a Miyako blender, and then made into a ball shape, and subjected to boiling water (Purnomo and Rahardiyan, 2008). The binary reference meatballs and fresh meat were stored at −20°C immediately after preparation until being used for DNA extraction.

DNA extraction: The extraction of DNA was performed according to Rohman et al. (2017) by weighing accurately 200 mg of samples (homogenized meat or meatballs) into 2 mL sterile reaction tubes. The samples were added with 800 μL buffer lysis (Tris HCl pH 8, EDTA pH 8, Na-acetate pH 5.2, NaCl, 1% SDS), 30 μL proteinase K (20 mg/mL), and 200 μL buffer lysis. This mixture was then homogenized using vortex (Barnstead, USA) for 5 min and incubated at 55°C for 1 hour using a water bath (IK HB 10, Medford, USA), with occasional shaking for every 15 min. The suspension was centrifuged (5 min, 13,000 gm) with microcentrifuge (Sartorius 3-30K Sigma, Jerman). The the supernatant (400 μl) was taken and added with 200 μL cold phenol (Merck, Darmstadt, Germany). The mixture was then shaken with a shaking incubator (Biobase, China) for 30 min. Furthermore, the suspension was then subjected to centrifugation at 13,000xg for 10 min. The supernatant was taken, added with chloroform (1:1 v/v) and subjected to centrifugation at 13,000 x g for 10 min. DNA was sedimented with Na-acetate 2.5 M pH 5.2 (1: 0.1 v/v) and cold ethanol absolute (1:2 v/v). The DNA was washed with 250 μL ethanol 70%, dried, and added with 50 μL buffer of Tris-EDTA consisting of 10 mM Tris HCl pH 8 and 1mM EDTA. The extraction was performed in duplicate.

Electrophoresis gel agarose for DNA qualitative analysis: The extracted DNA was analyzed qualitatively using electrophoresis in 0.8% agarose gel in TBE buffer 1X (boric acid, tris base, 0.5M EDTA pH 8) for 60 min at 90 V using electrophoresis system (i-Mupid J Cosmo Bio Co, Tokyo, Japan). The agarose gel was stained with GelRed™ (Biotium, Fremont, CA, USA) and visualized under UV light. The digital image was obtained using GBOX-Chemi-XRQ gel documentation system (Syngene, Synoptics Ltd., England) (Rahmawati et al., 2016).

DNA quantification and evaluation of DNA purity: The extracted DNA from evaluated samples (2 μL) was quantified using NanoVue™ Plus Spectrophotometer (GE Healthcare, Buckinghamshire, UK). The concentration of DNA was evaluated by measuring absorbance values at 260 nm in which 1 absorbance unit corresponds to 50 ng/μL of double-stranded DNA. The purity of extracted DNA was determined relying on the ratio (R) of the absorbance values at wavelengths of 260 and 280 nm. The R values in the range of 1.7–2.0 were accepted.

Real-Time PCR analysis: The extracted DNA samples were amplified by real-time PCR according to the procedure given by the manufacturer. In reaction tube, 10 μL Thunderbird Toyobo SYBR Green (TOYOBO CO., LTD., Osaka, Japan), 7 μL nuclease-free water, 1 μL of 0.06 μM forward primer, 1 μL of 0.06 μM reverse primer, and 1 μL of 75 ng extracted DNA sample were mixed and subjected to real-time PCR instrument (CFX96 Touch Real-Time PCR Detection System, Biorad USA). The following thermocycling program was used: initial denaturation at 95°C for 3 min in 1 cycle, then 95°C for 15 sec in 40 cycles, annealing at 60.7°C for 15 sec, and amplification at 72°C for 45 sec. The collection of the fluorescence signal was automatically performed at the end of each cycle. For the melting curve analysis, the temperature was increased by 0.5°C in the range of 65°C–95°C. Data were collected and processed using software of CFX Maestro™ included in real-time PCR instrument.

Validation of real-time PCR: Real-time PCR using designed primer was validated by determining several characteristics performances intended to its purposes which included primer specificity, sensitivity calculated as relative and absolute detection limit, efficiency value, and repeatability test according to CAS (2010).

Application of the validated method for analysis of commercial samples: The validated real-time PCR method was further applied for the analysis of DNA of meat species present in commercial meatballs obtained from several markets in Yogyakarta.
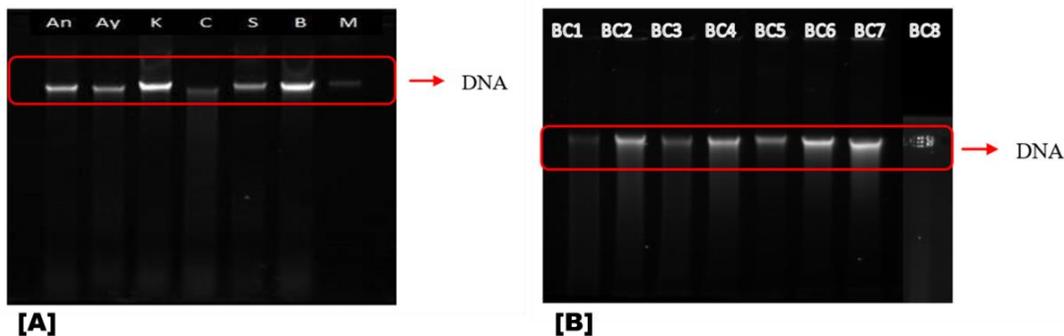
RESULTS AND DISCUSSION

In this study, the primer D-loop 443 was used to amplify a D-loop mitochondrial DNA fragment (145-bp). The primer was designed using online software of Integrated DNA Technologies from NCBI. Based on the *in-silico* results using BLAST tool in the URL address of https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome, the selected primer showed high specificity in *Sus Scrofa*/wild boar, as demonstrated by sequence similarity search and no cross-reactivity was observed with DNA from other species (**Figure 1**). But, D-loop 443-F primer depicted a similar sequence in *Sus scrofa domesticus* (Accession Number: NC_01209), although there were two mismatch bases and the length of the product was too long (16770-bp). In addition, D-loop 443-R had no similar sequence against the other species.

The primer was used to amplify DNA target in WBM, reference meatball containing WBM, and commercial meatballs. DNA extraction was performed using the ethanol-chloroform method as in Sambrook et al. (1989). The purity of the extracted DNA was checked by measuring isolate containing DNA at wavelengths of 280 and 260 nm. The purity index of all DNA isolates ranged from 1.77 to 1.96 for raw meats, and from 1.80 to 2.17 for reference meatballs corresponding to DNA yields of 783.5–3416 ng/μL and 324.884–985.27 ng/μL, respectively. This high purity of raw meat DNA extracts than the reference meatballs DNA extracts suggested that the exaggerated of heat used during meatballs processing made the purity and concentration of DNA in reference meatballs to be lower than that in raw meat. The quality of DNA extracted from the raw meat of seven species and reference meatballs from a binary mixture of WBM–
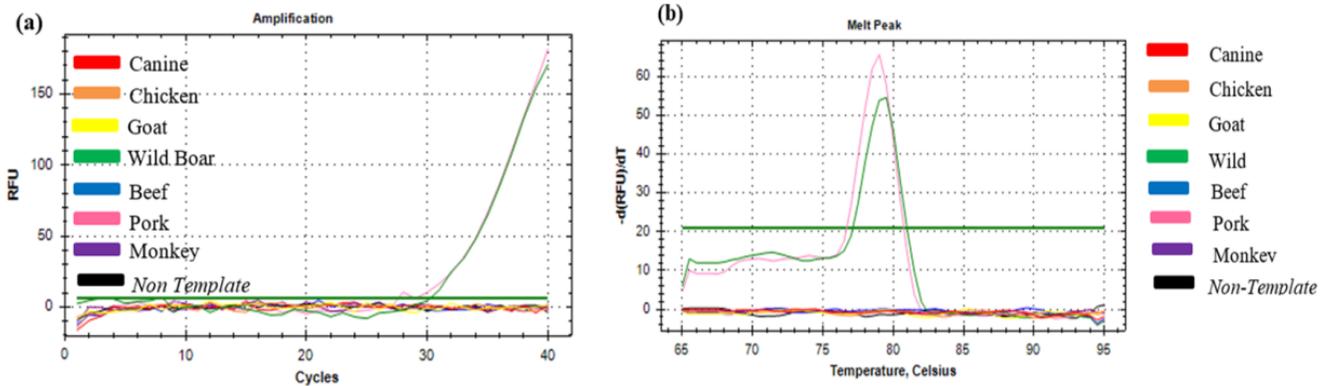
beef was observed under UV light using agarose gel electrophoresis. The results indicated that DNAs from all raw meats (dogs, chickens, goats, wild boars, cows, pigs, and monkeys) and DNA extracted from reference meatballs have been isolated and was not degraded during the excessive extraction protocol and heat, as showed in **Figure 1**.

For quantitative analysis, primer D-loop 443 in combination with real-time PCR analysis was validated by determining several parameters to assure that the method was fit to its purpose. To verify the primer specificity, the concentration of wild boar-specific primer used in this study was 10-fold lower (0.06 μM) than that as recommended in the protocol of fluorescent dye Thunderbird toyobo SYBR Green because in this study, the minimum conditions in which real-time PCR was still able to amplify the small amount of DNA in fresh meat and in meatball products was optimized. The annealing temperature of real-time PCR was also optimized, and that of 60.7°C was used as annealing temperature due to its capability to provide a specific and optimum response of amplification (**Figure 2**).
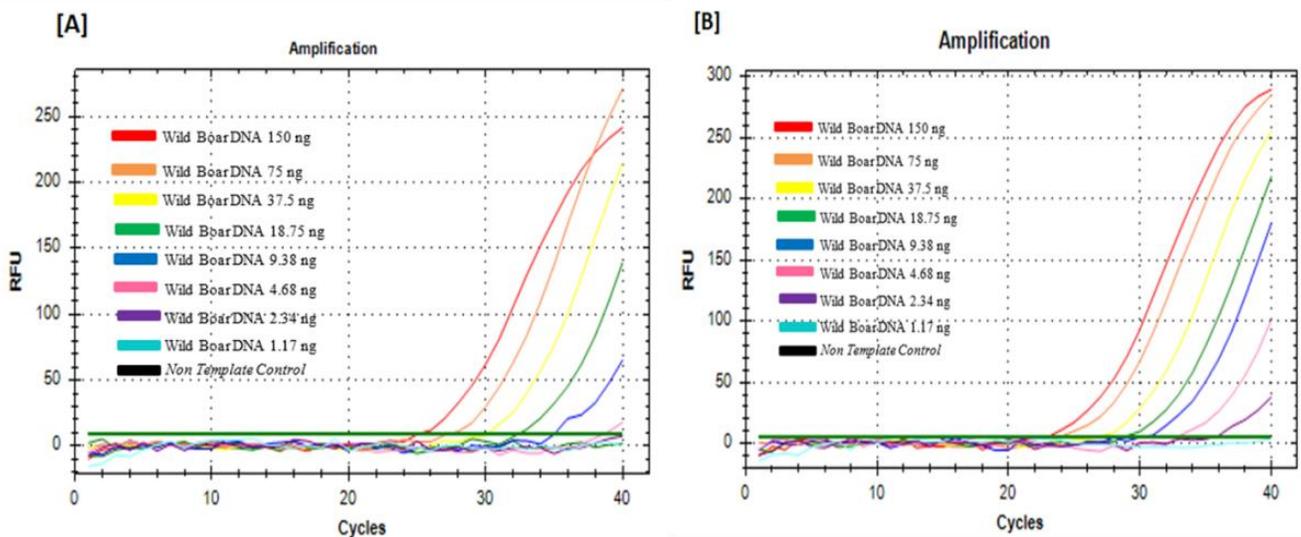
D-loop 443 primer had an amplicon size of 145 bp (Accession Number: KM016443) on the mitochondrial D-loop region. Raw meat samples of beef, chicken, goat, dog, pork, and monkey were used to confirm that the primers were WBM specific. As shown in **Figure 2A**, the cycle threshold (Ct) value of WBM used as the positive control was 30.29, whereas no amplification was achieved with DNA extracted from non-target animal species (beef, chicken, goat, dog, and monkey). Using denaturation curves, it is enabled to calculate melting temperature (Tm) and to verify the presence of unwanted DNA fragments, showing characteristic Tm of 79.50°C
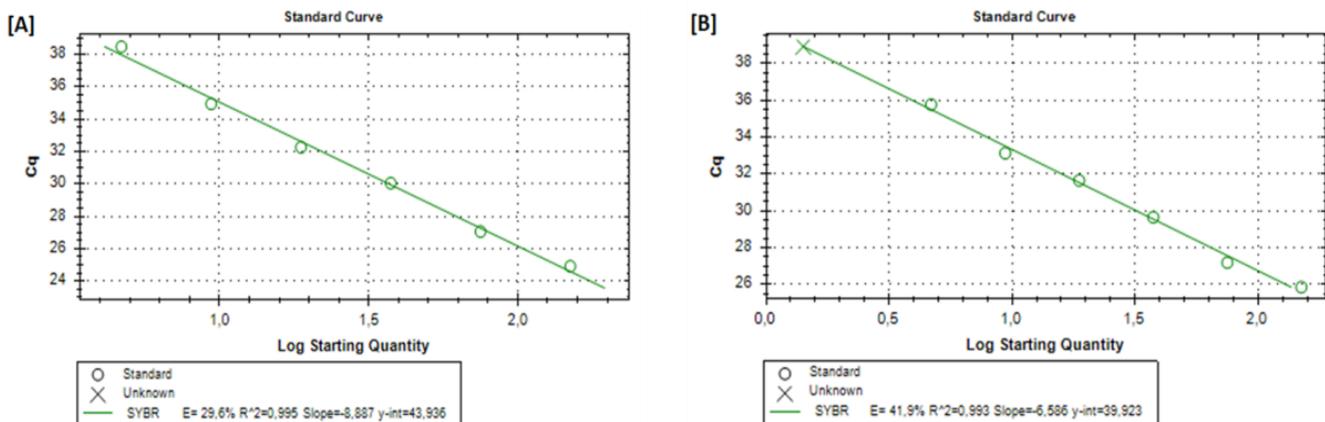

Figure 1. [A] Agarose gel electrophoresis of DNA extracted from fresh meat of canine (An), chicken (Ay), goat (K), wild boar (C), beef (S), pork (B), and monkey (M) on the agarose gel 0.8%; **[B]** agarose gel electrophoresis of DNA extracted from reference meatballs containing **w**ild boar at concentrations of 100% (BC1), 75% (BC2), 50% (BC3), 25% (BC4), 10% (BC5), 5% (BC6), and 1% (BC7), as well as meatball containing beef 100% (BC8).
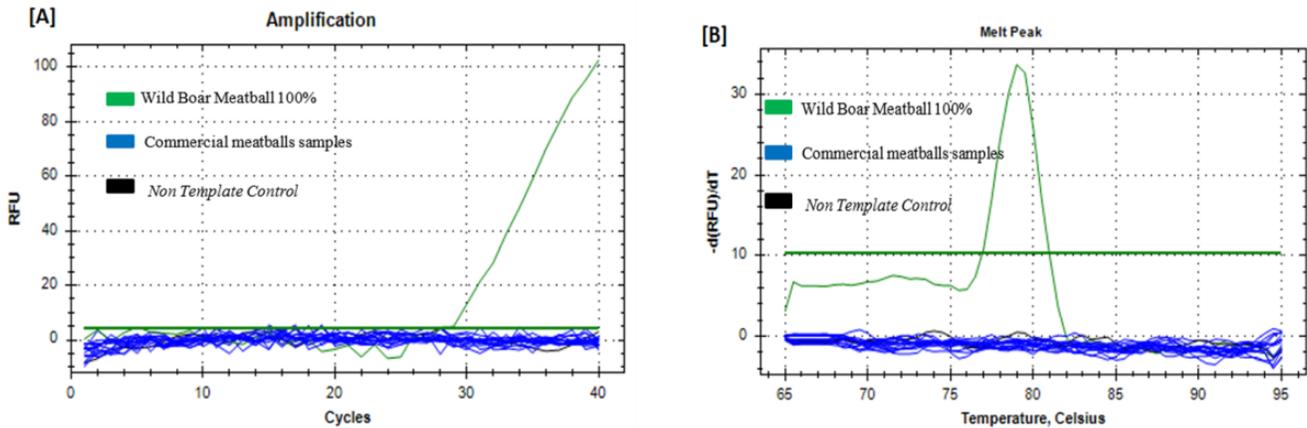
Figure 2. The specificity assay of primer D-loop using raw meat wild boar DNA at 60.7°C. **(a)** amplification curve peak and **(b)** melting curve peak.

Figure 3. The sensitivity test for determining of limit of detection (LoD) of DNA extracted from fresh wild boar meat **[A]** and that extracted from meatball containing 100% wild boar meat **[B]**.

Figure 4. Standard curves for linear relationship between Ct values (y-axis) vs the $\log_{10}$ of the copy number of (DNA) extracted from fresh wild boar meat **[A]** and that extracted from meatball containing 100% wild boar meat **[B]**, used for calculating efficiency of amplification (E) and limit of detection.

Figure 5. The amplification results of DNA extracted from commercial samples, positive sample of meatball containing 100% wild boar meat and no template control. **[A]** Amplification Curve; and **[B]** Melting curve.

for WBM (**Figure 2B**). However, this primer also provided an amplification response to pork DNA (*Sus scrofa domesticus*). The location of homolog between D-loop 443 primer and sequence DNA of pork (Accession Number: NC_01209) was proven in-silico using BLAST with product length 16770 (**Figure 4**). Wild boar (*Sus scrofa*) and pork (*Sus scrofa domesticus*) are animals with the same species namely *"Sus scrofa"*. Nonetheless, there is a slight Tm difference of 0.5°C among them (79.50°C for wild boar and 79.00°C for pork). In the case of non-halal meat, this primer gave an advantage due to its ability to detect the existence of a family of *Sus scrofa*. Therefore, either wild boar or pork DNA found in processed food products can be detected using D-Loop 443 primer.

The sensitivity of the real-time PCR system using D-loop 443 primer was determined by the amplification of DNAs extracted from fresh meat and 100% reference meatballs of wild boar, serially diluted (150; 75; 37.5; 18,75; 9,375; 4,6875; 2,34375; and 1,17188 ng /µL) to determine the smallest concentration of DNA could be detected in a sample (Huebner et al., 2001; CAS, 2010). The detection limit of WBM DNA found was 2.34 ng/µL, twice higher than that of DNA extracted from100% reference meatballs of wild boar (**Figure 3**). Standard curves were constructed from Ct values of the real-time PCR assay, with the good linear relationship between Ct values vs the $\log_{10}$ of the copy number. The correlation coefficients (R^2) of the linear regression curve for raw meat wild boar DNA was 0.995, with the slope -8.887, and y-intercept 43.936 indicating good correlation and high degree accuracy while the efficiency of amplification (E) was 29.6% (**Figure 4A**). The value of R^2 has met the qualitative and quantitative test criteria of the real-time PCR method while the PCR efficiency value

is less than the recommended criterion ($R^2 \geq 0.98$, and E=90–110%) (CAS, 2010). A good linear regression was also obtained from the standard curve of the amplification reaction of DNA extracted from 100% boar meatballs (**Figure 8b**), with high correlation coefficient (R^2) value 0.993, the slope of -6.586, and y-intercept of 39.923, so that the curve satisfied good linearity criteria. However, the efficiency value (E) obtained was 38.8%, which is smaller than the recommended efficiency range of 90%–110%.

The precision of real-time assay using D-loop 443 primer was evaluated using intermediate precision from three different days of measurement. The coefficient of variation (CV) Ct values at six replicates found were 4.87% and 4.01% for DNA extracted from fresh WBM and that extracted from meatball containing 100% WBM, respectively. Both CV values have met the criteria recommended for PCR method, *i.e.*, CV≤25% (Huebner et al., 2001; CAS, 2010). The validated method was subsequently used for the analysis of DNA in commercial meatball samples. Fifteen samples from different districts in Yogyakarta, Indonesia were evaluated using steps as in validated method. From all tested products, there were no declared labels related to the types of meat. Amplification results in **Figure 5** revealed that only positive control (DNA extracted from meatballs containing WBM) was amplified, which indicated that the tested commercial meatball samples did not contain WBM in their formulation.

CONCLUSION

D-loop 443 primer using real-time PCR at an optimum annealing temperature of 60.7°C was able to identify the

presence of wild boar DNA and also pork DNA. The developed method can be used for the analysis of non-halal meats (pork and WBM), but it could not differentiate the both types of meats. The method could be used as a standard method for authentication analysis of meatball products, thus, supported Indonesian Act No. 33, 2014 on Halal Products Assurance.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

The authors have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHORS' CONTRIBUTION

RLA, DR, and NWP performed research activity, compiled data, and prepared manuscript. Sis and AR designed research, analyzed data, and prepared manuscript.

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