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The employment of real-time polymerase chain reaction coupled with species-specific primer for analysis of wild boar meat for halal authentication

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Abstract

Wild boar meat (WBM) is considered one of the non-halal meats which are typically used as meat adulterant. WBM is non-halal meat which can be found in meatballs and sausages products, therefore, the detection of WBM is very essential for halal authentication analysis. The objective of this study was to use real-time polymerase chain reaction combined with species-specific primers. Primers were in silico designed and the selected primers were checked for their specificity in several DNAs extracted from corresponding raw meats. The specificity of real-time PCR using primers targeting ND5, Forward: TCGCCTCACTCACATTAACC and Reverse: GGGACTAGGCTGAGAGTGAA was tested against DNA templates extracted from different meat species. The annealing temperature (Ta) was also optimized to get Ta with optimum amplification. Some characteristic performances which were related to quantitative analysis using real-time PCR were also determined including detection limit, efficiency value and repeatability. The results showed that the primer used could amplify DNAs extracted from WBM and pork. The capability of primers to only amplify WBM and pork is significant because both types of meat are non-halal. Furthermore, real-time PCR using ND5 primer is capable of amplifying DNAs as low as 5 ng. Real-time PCR using species-specific primer provides reliable results for the authentication of halal meats and can be used as routine monitoring in halal authentication analysis.

1. Introduction

In recent years, the awareness of Muslims about the meat they eat is increasing. For the Muslim community, the consumptions of halal meat and halal meat-based food products such as meatballs and sausages are a must (Nakyinsige *et al*., 2012). Due to the price discrepancy, unethical producers of meat-based food products try to substitute or adulterate halal meat (for example beef) with non-halal beef to gain economic benefits. In Indonesia, some non-halal meats such as pork, wild boar meat, canine meat, and rat meat can be found in certain markets (Erwanto *et al*., 2018; Aina *et al*., 2019; Kurniasih *et al*., 2020). In addition, these non-halal meats are used as halal meat adulterants to reduce production costs. Even, some non-halal meats such as rat meat can be found freely from the farmers (Ali *et al*., 2015). As a consequence, some analytical chemists continuously propose and develop analytical methods capable of detecting non-halal meats specifically (Lubis *et al*., 2016).

Analysis of halal meats can be carried out using some biological biomarkers present in meat such as protein, lipid, and DNA, therefore some analytical

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methods based on lipid, protein and DNA are reported in scientific publications. Protein-based methods such as liquid chromatography hyphenated with mass spectrometer (LC-MS) in combination with chemometrics (Sarah *et al*., 2016), electrophoresis (Kim *et al*., 2017), enzyme-linked immune sorbent assay (ELISA) using specific antibody (Zvereva *et al*., 2015) as well as lipid based methods such as FTIR spectroscopy (Kurniawati *et al*., 2014), differential scanning calorimetry (Guntarti *et al*., 2017), electronic nose (Nurjuliana *et al*., 2011), and gas chromatography-mass spectrometry in combination with chemometrics (Rohman and Fadzillah, 2018) have been widely reported for halal authentication of meat-based food products. However, some of these methods lack specificity and involve extensive sample preparation such as derivatization in GC-MS analysis, therefore, DNA-based methods are currently accepted as the method of choice for the analysis of non-halal meats (Chen *et al*., 2010).

DNA-based methods using polymerase chain reaction (PCR) and its development such as real-time PCR and multiplex offered some advantages for meat identification. During heat processing and extensive food processing, DNA in food products is more stable than proteins, although DNA can be fragmented during heating, modern DNA-based techniques still allow the identification of DNA from different species present in food samples. Real-time PCR techniques are especially suitable for meat identification in heated food products because the small fragments of DNA can still be amplified and identified. PCR-based methods also offer highly specific methods for the detection of low or even trace amounts of DNA extracted from non-halal meats. The presence of inhibitors can influence the PCR results (Jonker *et al*., 2008). Due to the ability to directly examine and conduct a quantitative analysis of the amplification result using a DNA dye or fluorescence probe, RT-PCR is a more straightforward procedure to employ than conventional PCR (Widyasari *et al*., 2015; Yusop and Bakar, 2020). Besides, the experiment takes less time since real-time PCR results can be evaluated without gel electrophoresis (Erwanto *et al*., 2018; Yusop and Bakar, 2020).

Real-time PCR using species-specific primer has been successfully applied for the detection of non-halal meats such as identification of pork (Maryam *et al*., 2016), wild boar meat (Guntarti *et al*., 2017), canine meat (Kurniasih *et al*., 2020), rat meat (Widyasari *et al*., 2015), and monkey meat (Razzak *et al*., 2015). Guntarti *et al*. (2017) found that primer CytbAG3A at a specific annealing temperature can amplify specifically DNA from WBM with acceptable efficiency (105%). Aina *et al*. (2019) found that primer CYTBWB2-wb was specific to wild boar species with a detection limit of as low as 5 pg/μL. DNA bases of PCR amplification with these primers have a similarity of 93– 98% to the *Cyt-B* mt-DNA gene from several types of wild boar (Aina *et al*., 2020). D-loop 443 primer detected the presence of wild boar DNA and pig DNA using real-time PCR at an ideal annealing temperature of 60.7°C (Arini *et al.*, 2018). PCR techniques based on mitochondrial genes exhibit higher sensitivity compared to single or low copy nuclear DNA targets, therefore the ND5 mitochondrial gene was chosen as a target for porcine DNA detection (Raharjo *et al*., 2017). ND5 gene primer was the highest and most specific match primer in detecting pig DNA fragments when compared to the *Cyt-b* and D-Loop gene primers (Kusnadi *et al*., 2020). The primer ND5 has been developed and used as a benchmark for developing primers on NADH dehydrogenase (Kusnadi *et al.*, 2021). In this study, real-time PCR using species-specific primer targeting ND5 was used for identification and quantitative evaluation of DNAs from wild boar.

2. Materials and methods

Samples of raw meats of wild boar (*Sus scrofa*) were obtained from Sumbawa, West Nusa Tenggara Indonesia; beef (*Bos taurus*), lamb (*Capra hircus*), chicken (*Gallus gallus*), rabbit meat (*Leporidae*) rat meat (*Rattus novergicus*), and canine meat (*Canis lupus familiaris*) were obtained from Yogyakarta, Indonesia. All samples were collected in 2022 and kept at a temperature of -20°C.

2.1 Primer design

The species-specific primer for wild boar DNA was designed using NCBI – Primer BLAST software from the website NCBI, and ordered from PT Genetika (Indonesia). To choose the sequence, NCBI – Nucleotide using Genbank or Accession ID was applied. The selected primer was based on some criteria including % GC, melting temperature (Tm), primer length and self-dimer. The primer obtained was tested for its specificity in silico toward DNAs of *Bos taurus*, *Gallus gallus*, *Leporidae*, *Capra hircus*, *Canis lupus familiaris* and *Rattus novergicus* using NCBI BLASTn (Basic Alignment Search Tool-nucleotide). The primers selected (Table 1) are those complement to only *Sus scrofa*, and the primers pair were provided by PT Genetika Science Indonesia.

2.2 DNA extraction

Extraction of DNA in raw meat was performed according to Rohman *et al*. (2020) with slight modification. Two series of raw meats were finely

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Table 1. Three pairs of primers (forward and reverse) along with their characteristics selected from the primer designing process using NCBI – Primer BLAST software from website NCBI.

No	Primer	Length	Tm	GC%	Self-Complementary	Self-3' Complementary
1	Forward: ATTGTAGCTGGACTTCATGGAAC	23	58.73	43.48	4.00	2.00
	Reverse: TGTCCCGTAACCATTGACTGAA	22	59.63	45.45	3.00	3.00
	Product length : 80					
	(D-Loop) Genbank MK251046.1					
	https://www.ncbi.nlm.nih.gov/nuccore/MK251046					
2	Forward: TCGCCTCACTCACATTAACC	20	57.62	50.00	4.00	0.00
	Reverse: GGGACTAGGCTGAGAGTGAA	20	58.15	50.00	4.00	0.00
	Product Length: 139					
	(ND5) Genbank MK251046.1					
	https://www.ncbi.nlm.nih.gov/nuccore/MK251046					
3	Forward: CCTTCATAGGCTACGTCCTG	20	57.20	55.00	5.00	1.00
	Reverse: TCTACGAGGTCTGTTCCGAT	20	57.59	50.00	3.00	2.00
	Product Length: 106					
	(*Cyt-b*) Genbank MK251046.1					
	https://www.ncbi.nlm.nih.gov/nuccore/MK251046					

grounded using mortar and stamper. In a 1.5 mL-micro tube, 200 mg of samples were added with 0.7 mL of lysis buffer and 30 µL of proteinase K (20 mg/mL). This mixture was homogenized and then incubated at 55°C for 2 hrs. After that, the mixture was vortexed and added with 710 µL of phenol-chloroform-isoamyl alcohol. The mixture was subjected to centrifugation at 12000 rpm for 15 mins to form two layers. The supernatant obtained was taken and added with 2-propanol with the same volume as the sample volume (1:1). Then, the tubes were incubated in deep freezer at -80°C for 1 hr. The pellet was taken and washed with 250 µL 70% ethanol and dissolved in 50–200 µL TE buffer (10 mM Tris HCl pH 8 and 1 mM EDTA). DNA obtained was further checked using agarose gel electrophoresis and its purity was determined using absorbance ratio at 280 and 260 nm.

2.3 Evaluation of isolated DNA and determination of DNA purity

The extracted DNAs were evaluated using agarose gel electrophoresis (i-Mupid J Cosmo Bio Co, Tokyo, Japan). DNA isolated (3 µL) was added with 2 µL loading buffer, and analyzed using gel agarose 0.8% using TBE buffer 1× (each 1 L consists of 10.8 g, Tris base, 5.5 g boric acid and 4 mL EDTA 9.5 M pH 8.0), previously added with FloroSafe®. Electrophoresis was carried out using a current 100 V for 45 min. Gel agarose as a result of electrophoresis was visualized using a mini transilluminator (Bio-Rad, USA) and the obtained figure was documented. Determination of DNA purity and DNA concentration was performed using NANO-Quant SPARK TECAN. A-2.0 µL DNA isolate was measured using NANO-Quant to get the concentration of DNA (ng/µL) and ratio value of absorbance at 230 nm and 260 nm (A230/A260) as well as absorbance values at 260 and 280 nm (A260/A280). DNA is considered pure if R-value at 260 and 280 (A260/A280) 1.8-2.0.

2.4 Real-time PCR analysis

Analysis of DNA was carried out using Real-time PCR CFX96 (Biorad, USA). The composition of reagents (with a total volume of 10 µL) used for each real-time PCR running was: 3.5 µL Free Nuclease Water, 5 µL SensiFAST™ SYBR, 0.5 µL 1 µM ND5 Forward Primer, 0.5 µL 1 µM ND5 Reverse Primer (the final concentration of each primer is 0.05 µM) and 0.5 µL DNA template. The conditions of real-time PCR used were: initial denaturation at 95°C for 3 mins (1 cycle), denaturation at 95°C for 10 s, annealing temperature at optimized temperature for 15 s, extension or amplification at 72°C for 10 s, and elongation at 72°C for 5 min to stabilize the amplification product. The annealing temperature (Ta) of primers was optimized toward DNAs extracted from wild boar meat at the temperature range of 58°C – 60.2°C.

2.5 The evaluation of performance characteristics of Real-time PCR

The primer specificity of wild boar DNA was tested against DNAs extracted from raw meats of beef, lamb meat, chicken, rabbit meat, canine meat and rat meat at a final concentration of DNAs of 50 ng/µL using real-time PCR conditions explained in the previous section with optimum annealing temperature of 59°C. The sensitivity of ND5 primers was evaluated by diluting wild boar DNA templates into six different concentrations (10000, 1000, 100, 10, 5 and 1 pg/µL) and the cycle threshold (Ct) value was recorded. Linear regression correlating between log concentration of DNA and Ct-values was constructed to evaluate the method sensitivity. The repeatability of real-time PCR during amplification was evaluated by assessing the relative standard deviation (RSD) value or coefficient of variation (CV) of a cycle of quantification (Cq-values) at a concentration of DNA

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wild boar of 42.77 ng/µL.

3. Results and discussion

In order to examine wild boar meat for halal verification, the study set out to build a species-specific primer targeting the ND5 gene in conjunction with a real -time polymerase chain reaction (PCR). Three sets of primers (forward and reverse), as well as information about them, were chosen throughout the primer creation process using the NCBI Primer-BLAST program from the NCBI website (Table 1). The D-Loop, ND5, and *Cyt-b* genes in pig mt-DNA (*Sus scrofa*) were used to design the primers, which have shorter amplicon targets than the prior study (Kusnadi *et al*., 2020). When cycling periods are short, a longer amplicon could reduce amplification efficiency and affect how DNA amplification products separate using electrophoresis (Rohman *et al*., 2020). The DNA from the wild boar meat was specifically amplified using the design primers. In comparison to D-Loop and *Cyt-B* primer, the results showed that the ND5 primer produced greater amplification, yielding more copied DNA. According to Raharjo *et al*. (2017), PCR techniques based on mitochondrial genes exhibit higher sensitivity than single or low copy nuclear DNA targets, therefore the ND5 mitochondrial gene was chosen as a target for porcine DNA detection. ND5 gene primer was the highest and most specific match primer in detecting pig DNA fragments when compared to the *Cyt-b* and D-Loop gene primers (Kusnadi *et al*., 2020). As a result, only the ND5 primer was subsequently exposed to a validation experiment utilizing a real-time PCR instrument.

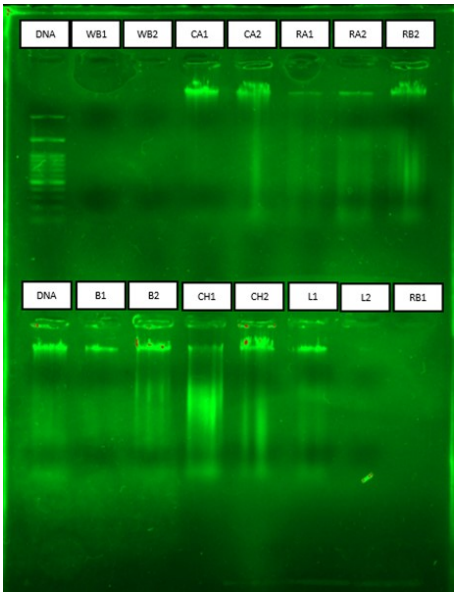

Figure 1. The gel agarose electropherogram of DNA isolated from different sources. DNA ladder (DNA), wild boar meat (WB1 and WB2)), canine meat (CA1 and CA2), Rat meat (RA1 and RA2), Rabbit meat (RB1 and RB2), beef (B1 and B2), Chicken (CH1 and CH2) and lamb meat (L1 and L2).

According to Rohman *et al*. (2020), with a little modification, the phenol-chloroform-isoamyl alcohol method was used to extract the DNA from raw meats. DNA extraction quality was assessed using electrophoresis on 0.8% agarose gel and UV imaging. According to the findings (Figure 1), all raw meats (beef, canine, chicken, rabbit, lamb and rat) had isolated DNA. A direct correlation exists between the amount of isolated DNA and the thickness of the resulting DNA band. The purity of the isolated DNA was then determined by measuring the solution's absorbance at 280 and 260 nm. Table 2 includes the extracted DNA concentration and its purity index. The purity index values between 1.7 and 2.0 were used to define pure DNA. Real-time PCR analysis can be performed using DNA templates with a purity index of 1.7 to 2.0. Table 2 shows that some samples have A260/A280 values between 1.8 and 2.0, while others have values above 2.0 that indicate the presence of RNA and others with values below 1.8 that indicate the presence of protein, carbohydrates and phenols in extracted DNA.

Real-time PCR device using primer ND5 was carried out to analyze the DNA. Initially, the annealing temperature (Ta) utilized for PCR analysis was optimized. The annealing temperature used was based on around theoretical melting temperature of 58 – 60.2°C using 30 cycles. Due to its capacity to deliver an optimal response of amplification, a temperature of 59°C was ultimately chosen as the ideal Ta with a quantification cycle (Cq) of 16.00 and melting temperature of 79.00°C (Figure 2a and b). Three criteria—specificity, sensitivity, and repeatability—were used to assess the real-time PCR's characteristic performance (Bustin *et al*., 2009). The specificity of the ND5 primer was tested using isolated DNA samples taken from lamb, rat, chicken, rabbit, wild boar, beef and dogs. The ND5 amplification curve was shown in Figure 3a, and it showed that the primer could only specifically amplify DNA from wild boar meat (Cq value: 15.30) and not DNA from other meats. It is possible to determine melting temperature (Tm) and confirm the existence of undesired DNA fragments using denaturation curves, with a characteristic Tm of 79.50°C (Figure 3b).

The ND5 primer was also tested on extracted DNA from pork (*Sus scrofa domesticus*), yielding an amplified response with Cq 14.61 and Tm = 78.50°C (Figure 4a dan b). The species "*Sus scrofa*" includes both pork (*Sus scrofa domesticus*) and wild boars (*Sus scrofa*). The melting temperature difference between wild boar and pork, which is 79.50°C vs. 78.50°C, is only 1.0°C. This primer not only provided a benefit in the case of non-halal meat because it could identify the presence of a *Sus scrofa* family (Arini *et al*., 2018) but also performed an

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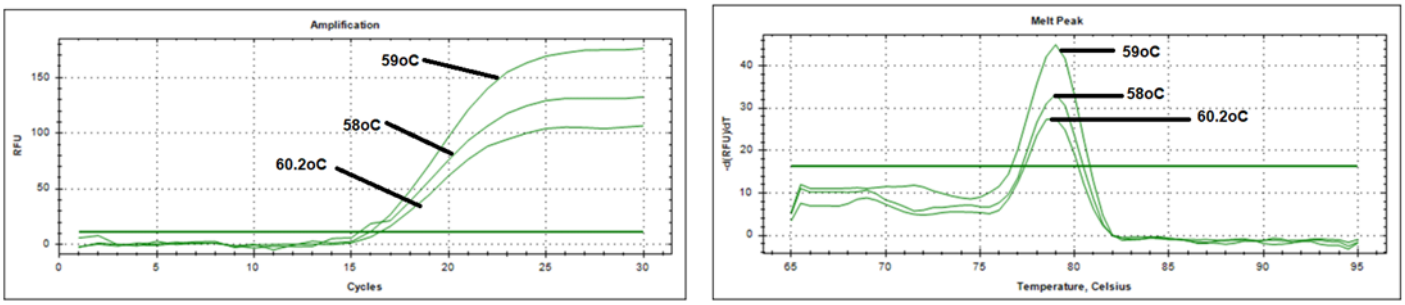
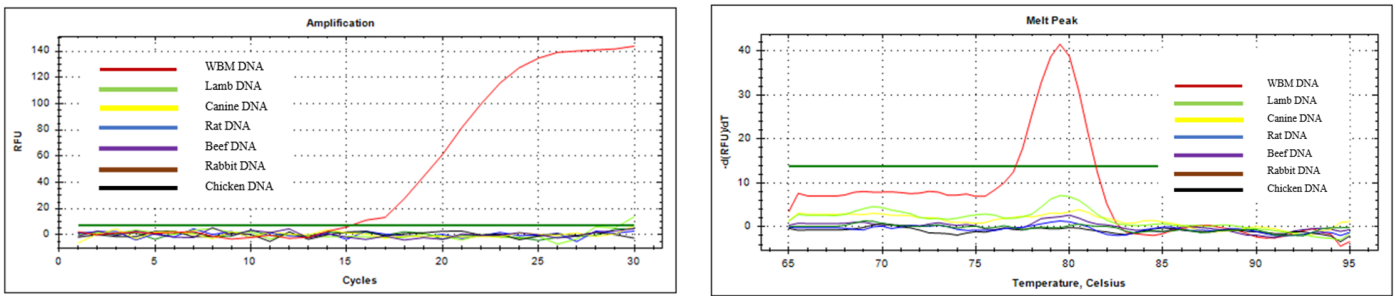
Table 2. The concentration of DNA along with DNA purity of DNAs extracted from raw meats.

Meat types	Concentration of DNA (ng/μL)	Purity index
Wild boar meat 1	19.62	1.85
Wild boar meat 2	42.77	1.79
Canine meat 1	3514.24	1.57
Canine meat 2	414.35	1.90
Rat meat 1	116.62	1.97
Rat meat 2	178.38	1.96
Rabbit meat 1	73.30	2.18
Rabbit meat 2	2499.25	1.99
Beef 1	258.08	1.95
Beef 2	78.93	2.00
Chicken meat 1	852.63	1.99
Chicken meat 2	3179.23	1.89
Lamb meat 1	883.68	1.92
Lamb meat 2	1210.22	1.91



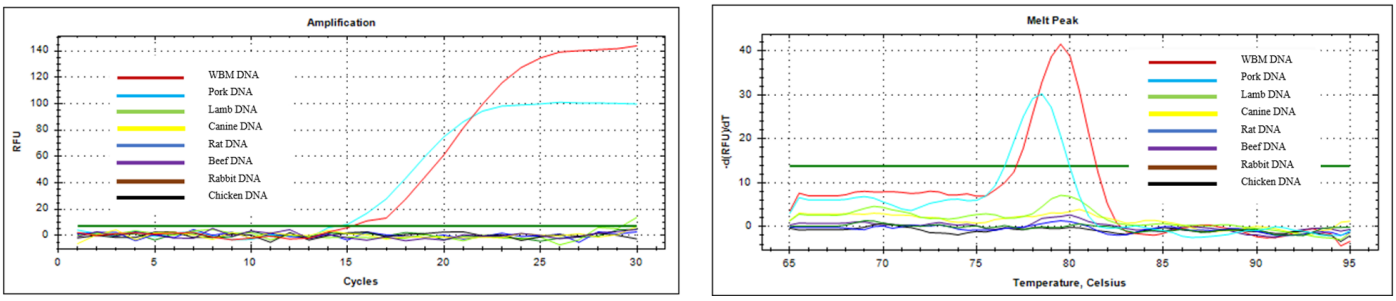
(a) (b)

Figure 2. The optimization of annealing temperature (Ta) of ND5 primers for analysis of DNA extracted from wild boar meat at temperature range of 58°C – 60.2°C: (a) Amplification curves and (b) Melting curves.



(a) (b)

Figure 3. Real-time PCR amplification of DNAs from different species (without pork) using ND5 primers intended for primer specificity: (a) Amplification curves and (b) Melting peak.



(a) (b)

Figure 4. Real-time PCR amplification of DNAs from different species (including pork) using ND5 primers intended for primer specificity: (a) Amplification curves and (b) Melting peak.

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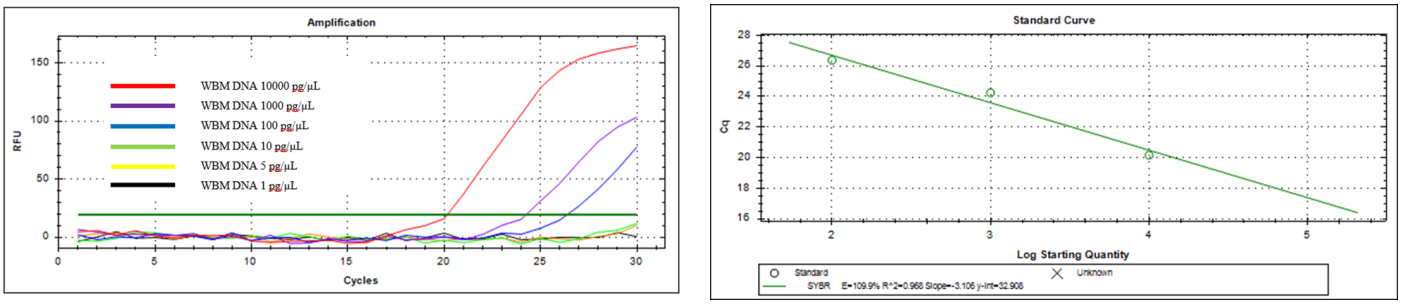

(a) (b)

Figure 5. Real-time PCR amplification using ND5 primers toward wild boar DNAs diluted at six different concentrations: (A) Amplification curves and (B) Calibration curves.

acceptable efficiency value. The sensitivity of the real-time PCR system using ND5 primer was determined by the amplification of DNAs extracted from wild boar meat serially diluted (10000; 1000; 100; 10; 5; and 1 pg / µL) to determine the smallest concentration of DNA that could be detected in a sample. The amplification profile of DNA extracted from wild boar meat with 6 different concentrations is shown in Figure 5a whereas the efficiency value is shown in Figure 5b. A correlation R^2 of 0.968 with an efficiency value of 109.9% and slope value of -3.106 was obtained. Standard criteria for efficiency, slope, and R^2 are, respectively, 90-110%, -3.9 to -2.9, and 0.98 (Broeders *et al*., 2014). The precision of the real-time assay employing the ND5 primer was assessed by a repeatability test. In six replicates of fixed wild boar meat DNA concentration (42.77 ng /µL), it was discovered that the coefficient of variation (CV) Cq values were 2.57%. This value complies with the PCR method's requirement for a CV of under 25% based on the Joint FAO/WHO Codex Alimentarius Commission (2010).

4. Conclusion

Primer targeting on ND5 in combination with real-time PCR is capable of identifying wild boar meat DNA. The primer also could amplify DNAs extracted from pork. The capability of primers to only amplify WBM and pork are advantageous because both meats are non-halal. Furthermore, real-time PCR using ND5 primer is capable of amplifying DNAs as low as 5 ng. The coefficient of determination (R^2) values obtained for the linearity test is 0.968 with an efficiency value of 109.9% with acceptable precision. Real-time PCR using species-specific primer provide reliable results for authentication of halal meats and can be used as routine monitoring in halal authentication analysis.

Conflict of interest

The authors declare no conflict of interest.

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