Specific Real-Time PCR Assay Targeting D-loop Gene and Short Amplicon Sequencing for Identification of Monkey Meat in Beef Meatballs

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Abstract: *Macaque monkey (*Macaca fascicularis*) meat (MM) has been reported to be consumed as meatball and soup products in Indonesia. MM is not allowed to be traded in Indonesia and is considered not halal meat; therefore, MM is not allowed to be consumed by Muslim communities. In this study, species-specific primer (SSP) targeting on mitochondrial displacement (D-loop) region coupled with real-time PCR assay was used to identify monkey meat in beef meatballs. The PCR product was also subjected to sequencing in order to ensure the adulteration practice of MM in beef meatballs. The primers were designed and subjected to* in silico *specificity test using BLAST. The used primers were: forward: 5'-TGACTCCCACCACATCCC-3' and reverse: 5'-GTGTGGAGCTAGAATATTGAACCG-3'. Real-time PCR assay using SSP targeting on mt-D-loop primers was capable of identifying MM in meatball products, specifically with a detection limit of 0.0078 ng corresponding to 1% MM in beef meatballs. The developed method can be proposed as the standard method for detecting MM in food products intended for halal authentication analysis, provided that DNAs are available in food products.*

Keywords: *authentication; D loop primers;* Macaca fascicularis*; real-time PCR; sequencing*

■ INTRODUCTION

A report by ABC News in 2010 indicated that macaque (*Macaca fascicularis*) meat (MM) is reported to be present in meatball products and soups in Indonesia [1]. MM is one of the non-halal meats which is not allowed to be consumed by the Muslim community. MM may be a serious problem for consumers because of pathogenic bacteria and viruses in monkey meat [2-3]. With the implementation of Indonesian Law No. 33 on Halal Product Assurance, the verification of halal labelling for halal products is a must. The European Commission legislation also requires the labelling of all ingredients in food products. Thus, the screening and tracing of food products containing non-halal meat is a concern, especially in Muslim countries. Some methods for the identification of meat-species products have been developed, including liquid chromatography [4], Fourier transform infrared (FTIR) spectroscopy in combination with multivariate data analysis [5], electronic nose and gas chromatography-mass spectrometer [6], and differential scanning calorimetry [7]. Some of these methods lack specificity and need extensive sample preparation. Therefore, methods that are specific for certain species are needed.

When the sample has been subjected to food processing, the DNA-based method, such as polymerase chain reaction (PCR), is preferred over protein-based methods, such as enzyme-linked immunosorbent assay (ELISA), which the protein-based method is not suitable for food products under high-temperature process [8]. DNA-based methods based using PCR are the method

of choice for species identification, as these methods have high stability and are unique [9-12]. Some methods such as PCR-restriction fragment length polymorphism (PCR-RFLP) [13-14], real-time PCR using species-specific primer [9,15], and PCR followed by sequencing [16-17] are reported for the identification of non-halal meats such as pork, wild boar, dog, and rat in several food products including meatballs, sausages, and jerk.

It is well known that species-specific primers (SSP) targeting specific genes are the deciding factor in PCR methods for meat identification. Primers derived from mitochondrial DNA (mt-DNA) are usually preferred in animal-based foods, especially on displacement (D-loop) genes [18-20]. The selection of primers based on mitochondrial DNA is due to some reasons in which most cells contain multiple copies of mt-DNA with a high mutation rate. In addition, primers targeting mt-DNA also provide good sensitivity with low detection limits and ease in its isolation for further analysis. In this study, a new SSP based on D-Loop mt-DNA was designed and validated for analysis of MM in meatball products.

Several previous studies have been reported for the identification of MM in food products. Ali et al. [21] and Rashid et al. [22] have developed using short Amplicon (120 bp) as a target by PCR-RFLP analysis. It is known that the primers producing short-sized PCR products can amplify the product more specifically and efficiently. However, there are no real-time PCR assays with short species-specific primers (< 100 bp) in public for MM authentication capable of detecting MM in real-time efficiently. In order to verify real-time PCR products, it is necessary to sequence the PCR products to ensure that PCR products are amplified by species-specific primers. PCR techniques and DNA sequencing proved to be powerful techniques for species identification [17]. In this study, very short primer (97-bp) targeting on D-loop was used for the identification of MM in meatballs, and the products obtained were sequenced using BigDye™ Terminator V 3.1 Cycle Sequencing Kit.

■ EXPERIMENTAL SECTION

Materials

A meat sample of macaque species (*M. fascicularis*) was acquired from The Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta, Indonesia. Other meats such as beef (*Bos taurus*), chicken (*Gallus gallus*), goat (*Capra hiscus*), pork (*Sus scrofa*), and dog (*Canis lupus*) were bought from local markets, while wild boar meat (*Sus scrofa domesticus*) was obtained from Palembang, South Sumatera, Indonesia. A total of 12 different commercial meatball products were bought from twelve different random local shops in Yogyakarta, Bantul, and Sleman, Indonesia. All samples were stored at −20 °C prior to use.

Instrumentation

The main instruments used are real-time PCR CFX96 (Bio-Rad, USA), UV-vis spectrophotometer Nanovue™ Plus (GE Healthcare, UK), GBOX-Chemi-XRQ gel documentation system (Syngene, UK), and AB Capillary Electrophoresis 3500 Genetic Analyzer (Thermo Fisher, USA).

Procedure

Preparation of reference meatballs

The reference meatballs were prepared using binary mixtures of MM and beef meat (BM) ranging from 0 to 100% (wt/wt), namely 0, 1, 5, 10, 25, 50, 75, and 100%, into a final weight of 20 g according to the previous study [23]. Each mixture was then added by 2 g tapioca flour and homogenized using a blender and shaped into a ball shape. All samples were cooked in boiling water (100 °C) for 20 min and stored at −20 °C before DNA extraction.

Isolation of DNA

DNA was extracted from approximately 200 mg of raw meats (beef, chicken, goat, wild boar, dog, pork, and macaque), reference meatballs, and commercial meatballs. Each sample was blended using a commercial blender, each with three replicates. DNA isolation was carried out according to Maryam et al. [9] with minor modifications. The sample was then added with 1 mL of lysis buffer (EDTA 0.1 M pH 8, Tris-HCl 50 mM pH 8, NaCl 0.1 M, SDS 1%) and 10 µL of proteinase K (20 mg/mL). Subsequently, the mixture was incubated at 60 °C for 2 h, with occasional stirring. A-1 mL supernatant was taken and extracted with phenol:chloroform and then finally precipitated with

ethanol 95% (1:1) and 50 µL sodium acetate 2.5 M (pH 5.2) prior to incubation for a night at −20 °C The precipitated DNA was washed in 70% alcohol, dried, and dissolved on 50 µL tris-EDTA buffer. The DNA sample was then stored at −20 °C before further analysis. The quality of DNA was analyzed by electrophoresis in a 0.8% agarose gel in 1× Tris-borate-EDTA buffer and stained by 1 µL GelRed™ (Biotium) at 50 V for 45 min. The concentration of DNA was estimated by UV-vis spectrophotometer at 260 and 280 nm, while a digital image was obtained using the GBOX-Chemi-XRQ gel documentation system.

Primer design

A pair of macaque-specific primers were designed using the online software Integrated DNA Technologies Primer3plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3 plus.cgi). Primer specificity was assessed through alignment analysis with D-loop genes of 7 meat species (beef: AB003801.1, goat: KM360063.1, chicken: KM096864.1, pork: AF034253.1, wild boar: MK251046.1, dog: NC_008092.1, and macaque: FJ906803.1) and gene of 2 plant species (onion: GU253304.1, tomato: XM004251454.1). This analysis was done using Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and oligonucleotide analyzer for dimer and hairpin analysis (http://sg.idtdna.com/site) [22]. Furthermore, the primer specificity was assessed using Clustal Omega to find out the total mismatch of the primers and provide a phylogenetic tree toward other species (https://www.geneious.com/prime/) [22,24-25]. The primers macaque targeting 97-bp D-loop gene were chosen in this work, namely forward: 5'-TGACTCCCACCACATCCC-3' and reverse: 5'-GTGTGGAGCTAGAATATTGAACCG-3'. These primers were purchased from Integrated DNA Technologies (IDT, USA) and were further analyzed with real-time PCR.

Analysis using real-time PCR

DNA samples (DNA extracted from raw meats, reference meatballs, and commercial meatballs) were amplified by real-time PCR CFX96 based on SSP-SYBR Green dye. PCR mixture containing 10 µL Ssofast™ EvaGreen® Supermix (Bio-Rad, USA), 7 µL Nuclease-Free Water, 1 µL of each primer (10 µM), and 1 µL extracted DNA sample (50 ng with total volume 20 µL) were put into a reaction tube. The cycle of real-time PCR was carried out as follows: initial denaturation as 1 cycle at 94 °C for 120 s, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55.5 °C for 30 s, and extension at 72 °C for 30 s, with the collection of fluorescence signal at the end of each cycle. For melting curve data, the temperature was increased by 0.5 °C in the range of 65 to 95 °C.

The sequencing of PCR amplicon

Approximately 2 µL PCR product was then separated in 2% agarose gel before being subjected to purification using GenepHlow™ Gel (Geneaid, Taiwan) with minor modification. Electrophoresis was done at constant voltage (50 V) for 45 min in 1× Tris-borate-EDTA buffer pH 8.0 and stained by 0.5 µL GelRed™ (Biotium). A marker HyperLadder™ 50 bp (Bioline, UK) was used as a size reference. The gel photo was taken using the GBOX-Chemi-XRQ gel documentation system. After that, the PCR product was sequenced using a 97-bp macaque-specific primer developed in this work as sequencing primer with BigDye™ Terminator V 3.1 Cycle Sequencing Kit. Sequencing data analysis was performed with AB Capillary Electrophoresis 3500 Genetic Analyzer. The result of sequencing was then processed using Molecular Evolutionary Genetic Analysis (MEGA) software to get the reverse complement [26]. Alignment analysis was done by Multiple Sequence Comparison by Log-Expectation (MUSCLE) (www.ebi.ac.uk/Tools/msa/muscle/) [27]. The minimal score of conformity between both sequences is 98% [28].

Evaluation of performance characteristics of real-time PCR

The performance characteristic of real-time PCR for quantitative analysis of DNA was carried out by determining specificity, sensitivity, and repeatability tests [29]. Specificity assay of the designed specific primer was confirmed by amplification of 50 ng DNA/µL of target species (macaque) and six non-target species (beef, chicken, goat, dog, pork, and wild boar) and negative control (no DNA template, NTC).

There are no cross-amplified species if only the macaque-species melting curve is present and none from the non-target species-melting curve. The sensitivity assay of pure meat was determined by serially diluting the macaque DNA to get DNAs with concentrations of 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 ng/μL. Meanwhile, the limit of detection (LOD) value was determined using DNAs extracted from the reference meatballs by serially diluting to get DNAs with concentrations of 25, 12.5, 6.25, 3.125, 1.56 and 0.78 ng/μL, respectively. These DNA concentrations were then used to make a standard curve (cycle threshold, Ct value versus log DNA concentration) to determine the amplification efficiency [15]. The repeatability test was carried out by investigating Ct values from six replicates, and then relative standard deviation (RSD) was computed. Replications were done using DNA 50 ng/μL for 3 consecutive days. The developed method was further used for the analysis of 11 commercial samples obtained around Yogyakarta, Indonesia.

■ RESULTS AND DISCUSSION

Quality and Quantity of DNA

In this work, the agarose gel images of the extracted DNA from raw meats are shown in Fig. 1. Meanwhile, the purity values (R-values) using spectrophotometer UV (A_{260}/A_{280}) from all the studied samples were between 1.7 and 2.0, and the concentration of DNA yielded from 303 to 3416 ng/mL. DNAs with R-values of 1.8–2.0 were considered pure and suitable for further analysis. The highest concentration of DNAs was extracted from raw chicken meat, while the lowest was from meatballs product which might be due to the prolonged heat and pressure processing. The treatment can degrade the genomic DNA [30].

Primer Design

The designed primer targeting d-loop DNA for the detection of macaque DNA in meat products, namely forward: 5'-TGACTCCCACCACATCCC-3' and reverse: 5'-GTGTGGAGCTAGAATATTGAACCG-3', has a length of 18 bp (forward) and 24 bp (reverse) producing amplicon of 97 bp. Previously, more than 350 bp amplicon have been targeted on macaque DNA. However, this amplicon can be degraded easily through food processing [22]. Therefore, in the present study, the designed species-specific primers produced a relatively small PCR product (< 150 bp) for reducing the degradation effect of DNA after exposure to high-temperature processes (boiled, autoclaved, and microwaved) [21-22]. The best criteria have been achieved for the designed primer, namely: GC content 30–80% [31], melting temperature must be lower than 2 °C to avoid hairpin and having a minimal 3'self-complement.

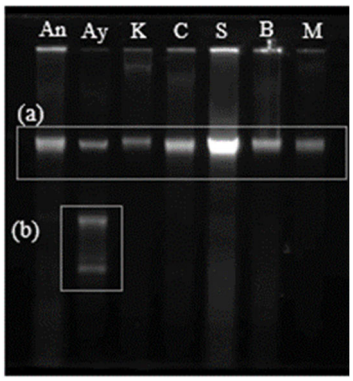

Fig 1. (a) Electrophoresis image of DNAs extracted from 7 raw types of meat, and (b) the degraded band from DNA contaminant. A_n = dog meat; A_y = chicken meat; K = goat meat; C = wild boar meat; S = beef meat; B = pork meat; and M = monkey meat

The designed primer was subjected to specificity evaluation using *in-silico* analysis to ensure that the developed primer would match perfectly with the specific target and have multiple mismatches with non-target species [22]. Firstly, the designed primer was aligned using BLAST from the NCBI to determine primer specificity against targeted nucleotide [32]. The primer BLAST result had 100% query coverage and an expectation value (E-value) of 1×10^{-5}. E-value ≤ 0.01 denotes homologous sequences with macaque D-loop gene [24], and this E-value is widely accepted for establishing the primer [33]. Secondly, the establishment of pair-wise distance and the phylogenetic tree was conducted with multiple aligned between the developed primer, 6 other meat species and 2 non-meat species. Perfect matching was observed only with *M. fascicularis*, and there was 6–24 nt (30–120%) mismatching with non-target species [34].

Pairwise distance obtained by the neighbor-joining method demonstrated distinct discrimination of the *M. fascicularis*-specific primers from all other species (Table 1) and 3D scatter plot (Fig. 2(a)). Phylogenetic tree (Fig. 2(b)) showed that the lowest distance was observed between macaque and cow, and the highest distance was found between macaque and onion, as indicated by the correlation of macaque and cow connected by the 2nd ancestor branch while macaque and onion were connected by the 1st ancestor branch (root). The high genetic distance indicated the unlikelihood of cross-species amplification in PCR running. Therefore, the designed primer targeting D-loop DNA, resulting very short amplicon length (97 bp) was used for the identification of MM in beef meatballs [35].

Real-Time PCR Analysis and DNA Sequencing

The optimization of annealing temperature is important to provide a temperature capable of increasing the primer specificity and rejecting nonspecific hybridization and producing the lowest quantification cycle (C_q) value and highest value of the ratio of fluorescence unit (RFU) [10]. The optimal annealing temperature obtained was 55.5 °C as determined through repeated running using gradient temperature in the range of 50–60 °C. At this temperature, species-specific primers for macaque DNA can produce the lowest amplification response and the highest melt peak Real-time PCR amplification without cross-reactivity with other non-target species DNA, as shown in Fig. 3. Melting Curve Analysis (MCA) enables to calculate of melting temperature (T_m of 79.00 °C) and verify the absence of unwanted double-stranded DNA fragments. Real-time PCR amplification using species-specific primers for macaque DNA at an annealing temperature

Table 1. Pairwise distance of the *M. fascicularis* against 5 animal species and 2 plant species

Species	*M. fascicularis*	*B. taurus*	*A. cepa*	*S. scrofa*	*C. hircus*	*G. gallus*	*C. lupus*	*S. lycopersicum*
M. fascicularis	0							
B. taurus	0.542	0						
A. cepa	1.473	1.237	0					
S. scrofa	0.867	0.255	1.969	0				
C. hircus	0.860	0.217	2.076	0.486	0			
G. gallus	1.133	0.917	1.594	1.412	1.332	0		
C. lupus	0.921	0.548	2.048	0.420	0.621	1.523	0	
S. lycopersicum	1.377	1.410	1.998	1.671	1.783	1.668	1.810	0

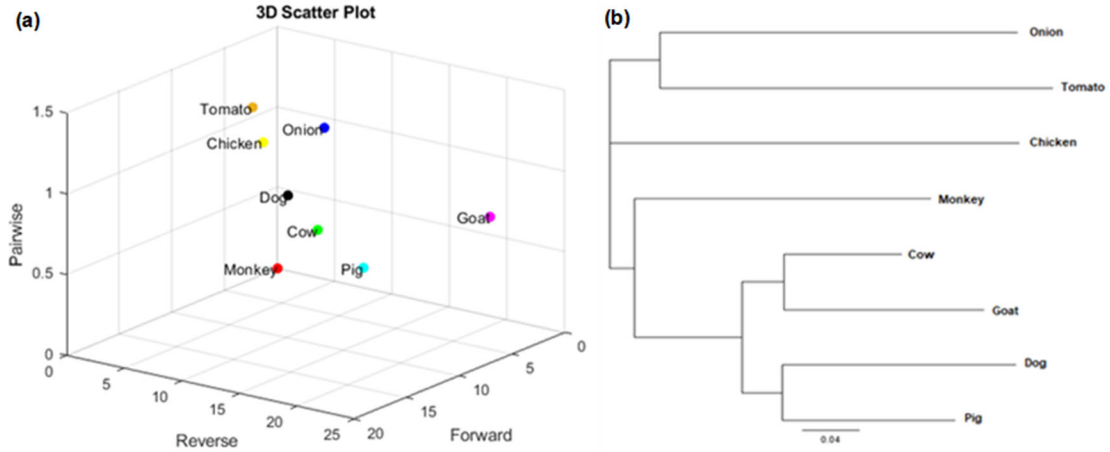

Fig 2. (a) The specificity analysis of macaque-specific primer pair against various animal species and plant species (3D scatter plot) and (b) phylogenetic tree

of 55.5 °C provided no cross-reactivity with other non-target species DNA which yielded C_q of 40 cycles for 100% MM and a negative result for other meat species.

The sequencing result of PCR product (97 bp) also confirmed that 100% similarity was observed only with the *M. fascicularis* D-loop gene (Fig. 4(a) and 4(b)). The alignment result indicated that the amplification using designed forward and reverse primers has 96.88% identic with gen D-loop mt DNA *M. fascicularis*. The high percentage of similarity showed that the designed primer in this assay specifically amplified 98–116 for forward primer and 171–195 reverse primer producing an amplicon with 96 bp length.

The Performance Characteristics of Real-Time PCR

The performance characteristics of real-time PCR for the analysis of macaque DNA in meatballs were carried out by evaluating the linearity, sensitivity, repeatability, and applicability of the method for the analysis of commercial samples. The linearity and sensitivity of the designed primer using real-time PCR for amplification of macaque DNA were determined using a serial dilution of DNAs at the concentration range of 50–0.39 and 25–0.78 ng of DNA extracted from raw meats and MM in beef meatballs, respectively. The values of C_q were plotted against the logarithms of DNA concentrations to make the standard curve. C_q represents the PCR cycle at which fluorescence reaches the threshold value. In this study, the developed method allowed real-time PCR efficiency (E) of 73.3% with a determination

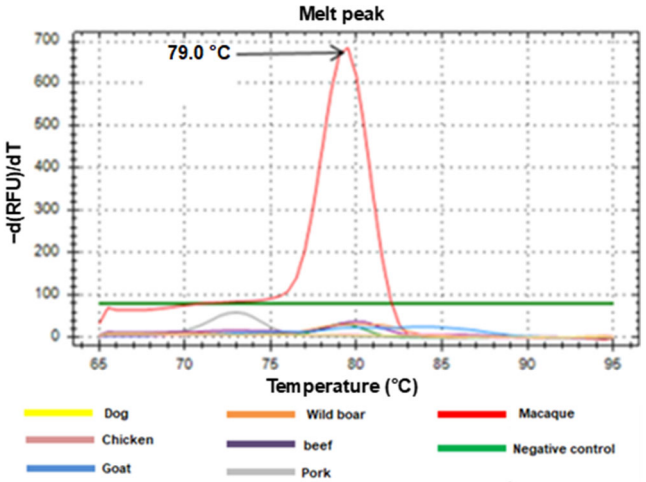

Fig 3. Melting peak curve of *M. fascicularis* DNA amplification product against 6 non-target species DNA at annealing temperature (T_a) of 55.5 °C

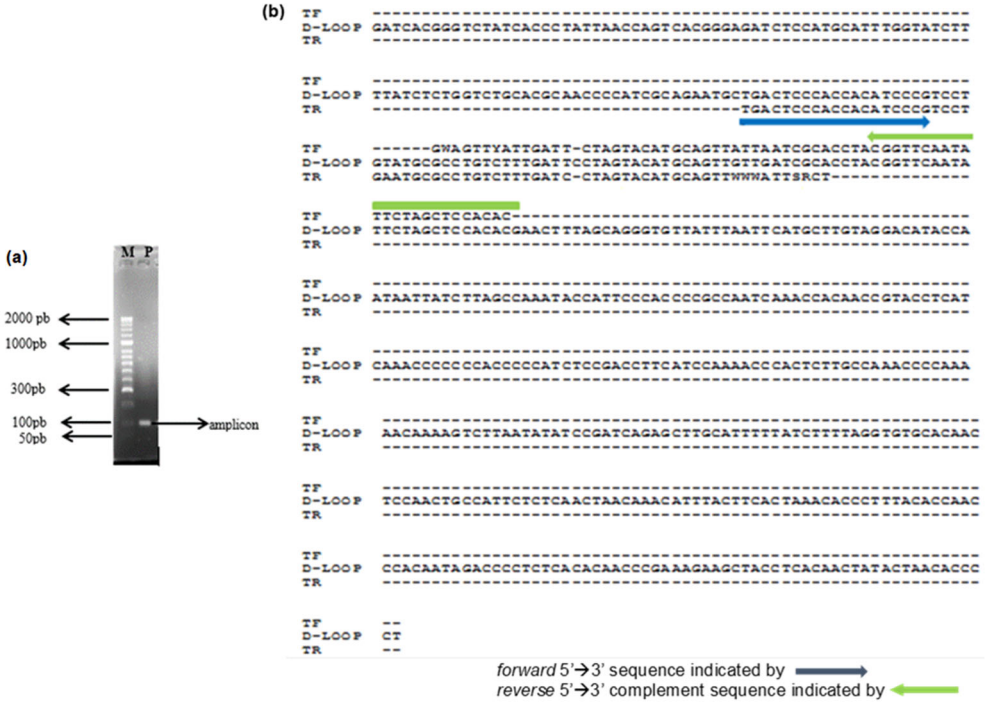

Fig 4. The amplification product obtained from (a) sequencing of *M. fascicularis* and (b) the alignment of sequencing product to mt D-loop region with CLUSTAL. M = marker; P = amplicon product

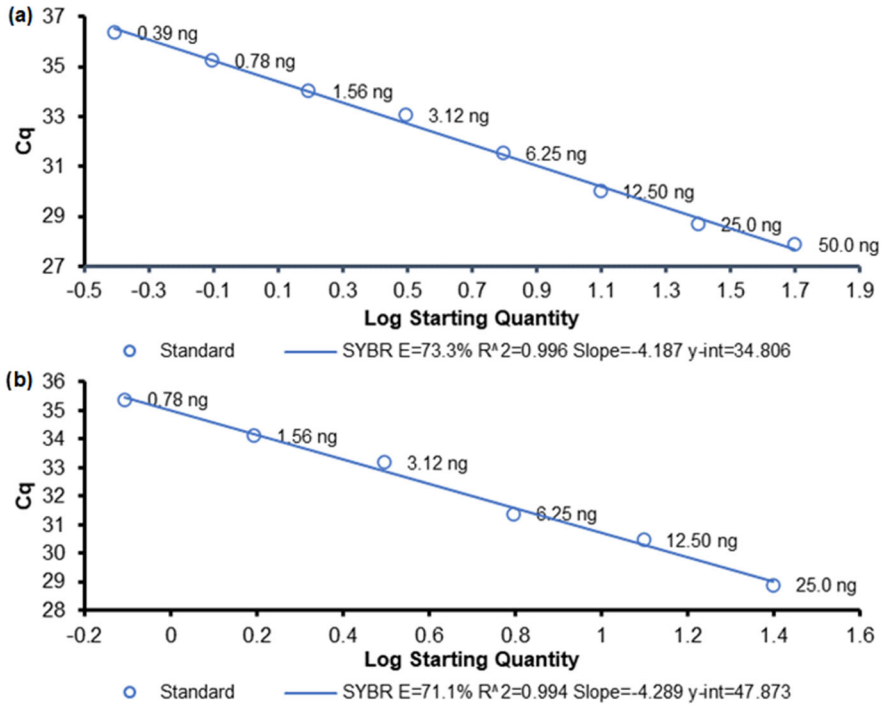

Fig 5. The standard curve of (a) 8 serial dilutions of DNA from raw MM and (b) DNA extracted from macaque meatball DNA standard curve of 6 serial dilution

coefficient (R^2) of 0.996 for DNA extracted from raw MM (Fig. 5(a)), E-value of 71.1% with R^2 of 0.994 for DNA extracted from 100% macaque meatball (Fig. 5(b)). Heat treatment during food processing had no effect on C_q value as at the level 0.78 ng, both DNA extracted from raw meat and macaque meatballs have the same C_q value [32]. According to Raymaekers et al. [33], the low score of % efficiency can be affected by PCR inhibitors, DNA degradation, amplicon length, secondary structure, and primer quality.

The sensitivity of the assay was constructed by determining the LoD of raw MM and MM spiked into beef meatballs. LoD values for real-time PCR using the designed primer was 0.0078 ng, corresponding to 1% MM in beef meatballs. Rashid et al. [22] reported the LoD of 0.1% (wt/wt) MM in a binary mixture with beef in meatballs using PCR RFLP. However, a previous study indicated that PCR RFLP is not suitable for meat admixture since the result of digestion can be false as a combination of miscellaneous restriction patterns for all possible species in the mixture [36].

The repeatability of the assay was established from replications of the amplification of DNAs extracted from meatballs at the same concentration (50 ng) of DNA for 3 d. RSD values obtained were 0.55, 0.86, and 1.81% in3 d, respectively, demonstrating a good reproducibility of the developed method. Analysis of MM in commercial meatballs revealed that the evaluated samples are negative for MM. The confirmation of meat types was further performed using a primer specific to beef (primer rRNA-12s mtDNA), and the results confirmed that 83.3% of meatball samples use beef and 17.7% of samples were not identified yet in terms of meat type used in meatball products.

■ CONCLUSION

The developed real-time PCR assay using specific primer targeting on mt-D-loop primers was capable of identifying MM in meatball products. The assay showed high specificity, and sensitivity, and can detect fraudulent MM in cooked meatballs without any challenging influence of processing conditions. The

specificity was proved using the sequencing method, assuring the assay could amplify specifically macaque species. This method can be used as a halal authentication technique for detecting MM.

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■ AUTHOR CONTRIBUTIONS

Dwiky Ramadhani Kurniawati: Methodology, Resources, Formal Analysis. Sismindari: Conceptual, Methodology. Rumiyati: Conceptual, Methodology. Fajar Setyo Wibowo: Conceptual, Methodology. Ni Wayan Pebriyanti: Methodology, Resources, Formal Analysis. Irnawati: Writing-review & editing. Abdul Rohman: Conceptual, Methodology, Resources, software, Formal Analysis, Writing-review & editing. All authors agreed to the final version of this manuscript.

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