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Development of real-time polymerase chain reaction for analysis of rat meat (*Bandicota bengalensis*) in beef meatballs for halal authentication

Abdul Rohman[1,2*], Hazza' Hammam Nawwaruddin[1,2], Hari Widada[3,4], M. A. Motalib Hossain[5], Marlyn Dian Laksitorini[1,2] and Dwi Lestari[6]

[1]*Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia*
[2]*Halal Center, Gadjah Mada University, Yogyakarta, Indonesia*
[3]*Post-Doctoral Fellow, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia*
[4]*School of Pharmacy, Faculty of Medical and Health Sciences, Universitas Muhammadiyah Yogyakarta, Yogyakarta, Indonesia*
[5]*Nanotechnology and Catalysis Research Centre (NANOCAT), University of Malaya, Kuala Lumpur, Malaysia*
[6]*Faculty of Pharmacy, Universitas Muhammadiyah Kalimantan Timur, Samarinda, Indonesia*

ABSTRACT

Background: Consumer awareness of food adulteration is increasing nowadays. Motivated by economic gain, unethical meat producers try to blend halal meat such as beef with non-halal meat like rat meat (RM).

Aim: This study aims to develop a real-time polymerase chain reaction (RT-PCR) analysis method to analyze the presence of RM in beef meatballs.

Methods: This research was carried out in the following stages: primer design, DNA isolation, analysis of DNA isolates, the optimization of primer annealing temperature, primer specificity test, sensitivity, and repeatability. The validated RT-PCR method was then used to analyze the marketed meatball samples.

Results: The result showed that the designed primer targeting on ND2 gene set rat mt-DNA (forward: ACTCCATATCTCTCACCATATTTCC; reverse: GGGTTAGGGTACTTAGGATTGTTAG), had good specificity at an optimal annealing temperature of 56.3°C over the other eight species. The developed RT-PCR method produces a limit detection value of 195.31 pg, coefficient of determination (R^2) for linearity of 0.983, amplification efficiency (E) of 100%, and CV value for amplification response of 1.8%. The result showed that the developed RT-PCR method did not detect the presence of RM DNA in eight marketed beef meatball samples.

Conclusion: The developed method meets the acceptance criteria for RT-PCR and can be used as a halal authentication method to identify the presence of RM in beef meatballs.

Keywords: Halal authentication, Rat meat, Meatball, RT-PCR, Species-specific primer.

Introduction

Meats and meats-based food products such as meatballs and sausages are popular foods in Indonesia and Asian countries. Meats are good sources of protein and minerals having important roles in human health (Cho *et al*., 2014). Halal food demand has experienced a substantial global increase, including in non-Muslim nations, due to growing awareness among people about the consumption of halal cuisine. Halal is not just connected to religion, but also linked to a healthy lifestyle (Alzeer *et al*., 2020). Various food products, including expensive meat items like beef meatballs and beef sausages, have the potential to be adulterated with non-halal substances such as pork, wild boar, dog, and rats by unscrupulous producers seeking economic gain (Selamat *et al*., 2021). The adulteration of halal meats, such as chicken and beef, with non-halal meats, such as pork, wild boar meat, canine meat, and rat meat (RM), is a significant issue. Muslims are strictly prohibited from consuming any food products that contain non-halal meats, even in minuscule quantities, as halal products have a zero-tolerance policy (Akbarzadegan *et al*., 2020). RM is considered non-halal due to its origin from animals with fangs. RM is linked to several diseases, such as leptospirosis, salmonellosis, Hantavirus pulmonary syndrome, Lassa fever, and tularemia. Since RM can be obtained freely from farmers, it has the potential to be used as a halal meat adulterant. Indonesian Drug and Control Agency in 2014 reported that RM was incorporated into halal meat-based food products of beef meatball beef sausages (Pebriana *et al*., 2017). Therefore, the availability of accurate and reliable methods for the detection of meat adulteration is very urgent.

***Corresponding Author:** Abdul Rohman. Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia. Email: *abdulkimfar@gmail.com*

Numerous analytical methods have been reported for screening and quantifying RM in food products including Fourier-transform infrared (FTIR) spectroscopy combined with chemometrics of pattern recognition and multivariate calibration (Guntarti and Prativi, 2017), gas chromatography-mass spectrometer (GC-MS) combined with partial least square-discriminant analysis through analysis of volatile components in RM, GC-MS combined with principal component analysis using variable of fatty acid composition (Salamah *et al*., 2022), and enzyme linked-immunosorbent assay or ELISA (Chen *et al*., 2020). However, these methods lack specificity. Hence, DNA-based methods, mainly based on polymerase chain reaction (PCR), are the method of choice for the detection and qualitative analysis of non-halal meats in food products (Rohman *et al*., 2021).

DNA-based procedures employing PCR are the method of choice or gold for extracting DNA from meat and meat-based foods (Lestari *et al*., 2024). PCR amplification is based on the hybridization of specific oligonucleotides to a target DNA and the synthesis of million copies flanked by these primers. The simplest PCR technique typically applied to detect non-halal meat products is the amplification of DNA fragments, followed by agarose gel electrophoresis for fragment size verification (Fajardo *et al*., 2010). Several PCR techniques have been successfully applied for the detection and confirmation of DNAs from non-halal meats. Species-specific primers (SSPs) targeting specific genes including mitochondria cytochrome-b, 12S rRNA gene, and d-loop are the most reported methods (Cahyadi *et al*., 2020; Kurniasih *et al*., 2020). The other PCR techniques used were Multiplex PCR assay (Ali *et al*., 2015) and real-time PCR (Amaral *et al*., 2017). The objective of this study was to design SSP targeting on the ND2 gene to detect RM in beef meatballs. The designed primer was also validated by determining several performance characteristics.

Materials and Methods

The raw meat samples used in this study including beef, goat, chicken, mackerel, pork, canine, frog, tree shrew, and Bengal rat were obtained from the local market in Yogyakarta, Indonesia. Eight marketed beef meatball samples were purchased from local food stalls in Yogyakarta, Indonesia. The designed primer for real-time PCR analysis was purchased from Genetika Science (Indonesia).

Primer designing

A set of primers targeting on ND2 gene in Bengal rat mt-DNA was designed using software from IDT DNA (https://www.idtdna.com/). The primer design was based on the complete genome sequence of Bengal rat mitochondrion with accession number NC_057104, retrieved from NCBI (http://www.ncbi.nlm.nih.gov). The primer specificity was analyzed *in silico* using basic local alignment search tool nucleotide (BLASTn) against Bengal rat (target) with 8 other species as a comparison (non-target) namely cow, goat, chicken, mackerel, pig, canine, frog, and tree shrew. Furthermore, an analysis was carried out on the possibility of primer secondary structure formation using the OligoAnalyser Tool software from IDT (https://www.idtdna.com/).

DNA isolation and quantification

The isolation of DNAs from raw meats and marketed beef meatball products was carried out using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen, Taiwan) according to the manufacturer's instructions with slight modifications. The DNA isolation method used in this study is a silica matrix-based method that consists of several steps, namely lysis, binding, washing, and elution. The DNA isolates were subjected to the purity and concentration assessment using NANO-Quant SPARK TECAN (Switzerland) at wavelengths of 260 and 280 nm. The purity of the DNA isolates was determined by the purity index (A_{260}/A_{280} ratio) in the range of 1.8–2.0.

Optimization of primer annealing temperature

The optimization of primer annealing temperature was carried out using a temperature gradient program, in the temperature range of 49°C–59°C, based on an estimation of primer annealing temperature that has been designed. The gradient temperatures used for the optimization were of 49.0°C, 49.7°C, 51.0°C, 53.6°C, 55.3°C, 57.3°C, 58.4°C, and 59.0°C. The eight levels of annealing temperature were determined automatically using real-time polymerase chain reaction (RT-PCR).

Analysis using real-time PCR

The DNA isolates were analyzed using PCR CFX96 (Bio-Rad, USA) with a total volume of reaction mix of 20 µl. The mixture composition consists of 10 µl of 2x SensiFAST™ SYBR® No-ROX Mix (Meridian Bioscience, USA), 1 µl of each designed forward and reverse primers (10 µM), 1 µl of template DNA with a concentration of 50 ng/µl, and 7 µl of nuclease-free water. The cycling condition used was as follows: polymerase activation at 95ºC for 3 minutes, denaturation at 95ºC for 5 seconds, annealing step at optimal annealing temperature for 30 seconds, and extension step at 72ºC for 20 seconds. For the melting curve analysis (MCA), the temperature was set at 65°C–95°C using a gradual increase of 0.5°C/5 seconds.

Validation and application of RT-PCR method

The real-time PCR method using a designed primer was subjected to qualitative RT-PCR validation testing criteria, which include primer specificity, sensitivity, amplification efficiency, and repeatability (Rohman *et al*., 2022). Primer specificity was confirmed by amplifying 50 ng/µl of extracted DNAs from the target (RM) and non-target species (cows, goat, chickens, mackerel, canine, pigs, frogs, and tree shrew) along with negative control or no template control (NTC). The sensitivity of developed RT-PCR was carried out by determining the limit of detection (LoD), the LoD as the lowest quantity of target DNA, which still can

be detected and amplified. The LoD value evaluation based on the amplification of isolate DNA of Bengal rat with serial dilution at a concentration of 25,000; 12,500; 6,250; 3,125; 1,562.50; 781.25; 390.63; 195.31; 97.66 pg. Amplification efficiency, coefficient of determination (R^2), and y-intercept were aimed to know the capability of the developed method in amplifying the DNA target. These parameters were determined using linear regression obtained by correlating the log value of the levels of DNA (x-axis) and cycle threshold (Ct) value (*y*-axis). The repeatability test was used to evaluate the precision of the developed method. The repeatability test of the developed RT-PCR method was evaluated by calculating the relative standard deviation (RSD) of the Ct value of the target DNA response from six replicates. The validated real-time PCR method was further applied for the analysis of the presence of rat DNA in 8 marketed beef meatball samples.

Ethical approval

Not needed for this study.

Results

Primer designing and evaluation of DNA isolates

In this study, the ND2 gene mt DNA of a Bengal rat was chosen as the target because, at the primer design stage, the targeted primers ND2 meets good primer design criteria in terms of primer profile, *in silico* specificity, and primer secondary structure. The primer profiles of ND2 set 4 were *forward*: ACTCCATATCTCTCACCATATTTCC, *reverse*: GGGTTAGGGTACTTAGGATTGTTAG which fulfilled the requirement parameters of good primer design, including primer length (18–30 bp), amplicon length (75–150 bp), Tm (54°C–65°C), GC content (40%–60%), and GC clamp.

Optimization of primer annealing temperature

The temperature of 53.6°C gives a high amplification response (relatively as high as 49.7°C) with a slightly higher Ct value than the temperature of 49.7°C. Therefore, an annealing temperature of 53.6°C was chosen as the optimum annealing temperature of the ND2 set 4 primer.

Validation of developed RT-PCR method

The LoD value for Bengal rat DNA was 195.31 pg, R^2 0.983, and efficiency value 100.0%. The repeatability test was carried out using one point on the standard curve at a DNA template concentration of 6250 pg. Ct values were in the range of 23.48–24.52, with an RSD value of 1.80%.

Discussion

Primer designing and evaluation of DNA isolates

One of the main objectives of this research is to design primer pairs that can identify Bengal rat DNA specifically. The primer design was based on the complete genome sequence of Bengal rat mitochondria. Mitochondrial DNA (mt-DNA) is used as an amplification target because it has several advantages. Mt-DNA has high variability in sequence among species, allowing adequate species identification in the sample mixture. The mt-DNA also offers a high copy number, so the amount of mt-DNA present in the sample mixture is also high and, thus, reducing the possibility of an insufficient number of DNA templates for RT-PCR testing (Maryam *et al*., 2016; Zhao *et al*., 2020). Furthermore, mt-DNA has good stability against degradation thus providing an advantage in the analysis of highly processed food products. *In silico* specificity test using BLASTn showed that ND2 set 4 had good specificity on the mtDNA of Bengal rat over the other 8 species (cows, goats, chickens, mackerel, canine, pigs, frogs, and tree shrews. Furthermore, analysis was carried out on the possibility of primer secondary structure using the OligoAnalyzer™ Tool software from IDT. Primer secondary structure analysis was carried out because the presence of primer secondary structure in the reaction can cause poor amplification results. For hairpins secondary structure, the T_m should be lower than the temperature at which the primer will be used. The analysis shows that the predicted hairpin structure has a very low Tm value, so it can be concluded that the ND2 set 4 primer meets the requirements for the secondary hairpin structure. Analysis of the primer secondary structure resulting from intermolecular interactions, in the form of self-dimers and hetero-dimers, is based on the value of the Gibbs free energy (ΔG). The Gibbs free energy (ΔG) value gives an indication of the strength of the secondary structure, which represents the energy required to break down the secondary structure. A lower value for ΔG indicates a higher level of stability in the secondary structure.The recommended ΔG acceptance value for self-dimers and hetero-dimers is −9. ND2 primer set 4 meets the acceptance criteria for the primer secondary structure analysis. The isolated DNA was then qualitatively and quantitatively analyzed by determining DNA's purity and DNA's concentration based on the measurement of UV absorbance at 260 nm (A_{260}) and 280 nm (A_{280}). The absorbance ratio of A_{260}/A_{280} which, indicates good purity of DNA isolates is within the range of 1.8–2.0. A ratio value less than 1.8 indicates the presence of protein contaminants in DNA isolates, while a ratio value more than 2.0 indicates the presence of RNA contaminants. Table 1 shows that the DNA isolates of all samples had a good purity index.

Optimization of primer annealing temperature

Optimization of the primer annealing temperature (Ta) is aimed at obtaining the optimum temperature at which the designed primer bonds with DNA templates at high efficiency and specificity. If the annealing temperature is too high, then the primers may not anneal to the target DNA. On the other hand, if the annealing temperature is set too low, the PCR primers can bind to the sequence outside of the target DNA (Sudjadi *et al*., 2016). Furthermore, Ta is generally 5°C lower than the primer melting temperature (Tm). Ta

optimization was performed at 49.0°C, 49.7°C, 51.0°C, 53.6°C, 55.3°C, 57.3°C, 58.4°C, and 59.0°C using the temperature gradient method available in the Bio-Rad CFX manager software. Figure 1 shows that ND2 set 4 primers gave the highest amplification response to Bengal rat mitochondrial DNA at 49.7°C (G) and 53.6°C (E).

The optimal annealing temperature is the temperature giving the lowest Ct value without the amplification of non-specific products such as primer-dimer (Biorad, 2023), which makes 53.6°C the optimum annealing temperature. On the other hand, the amplification curve data (Fig. 1a) also shows that as the temperature increases (at annealing temperature levels higher than 53.6°C), the amplification response becomes lower and more cycles are needed to reach the threshold. This indicates that, at these higher temperatures, it is more difficult for the ND2 set 4 primers to attach to the DNA template so that the number of PCR products formed and detected becomes less (Dolch *et al*., 2020). Furthermore, according to melt-curve analysis (Fig. 1b), the amplification at all temperature levels produces a single peak with a melting temperature (Tm) of 53.6°C, confirming that no primer dimer or non-specific products were identified during the optimization testing. The selection of an annealing temperature of 53.6°C rather than 49.7°C is also based on the principle that increasing the annealing temperature is known to increase the specificity (He *et al*., 2013). The annealing temperature of 53.6°C can give good specificity to Bengal rat mtDNA (target) over the other non-target species.

Table 1. The concentration and purity indexes of DNA isolated from raw meat samples.

Sample type	DNA concentration (ng/µl)	Purity index (A_{260}/A_{280})
Beef	217.0	1.82
Bengal RM	132.4	1.81
Chicken meat	698.8	1.92
Canine meat	309.8	1.84
Frog meat	339.9	1.99
Goat meat	273.9	1.85
Pork	134.2	1.80
Tree shrew meat	117.3	1.80
Mackerel meat	211.5	1.94

Validation of developed RT-PCR method

Validation of RT-PCR for analysis of Bengal rat mtDNA was carried out by determining several parameters, including specificity, sensitivity, amplification efficiency, and repeatability. The experimental specificity test aims to confirm that the ND2 set 4 primer meets good specificity for Bengal rat mtDNA. Primer specificity was confirmed by amplifying 50 ng of extracted DNAs from the target (Bengal rat) and

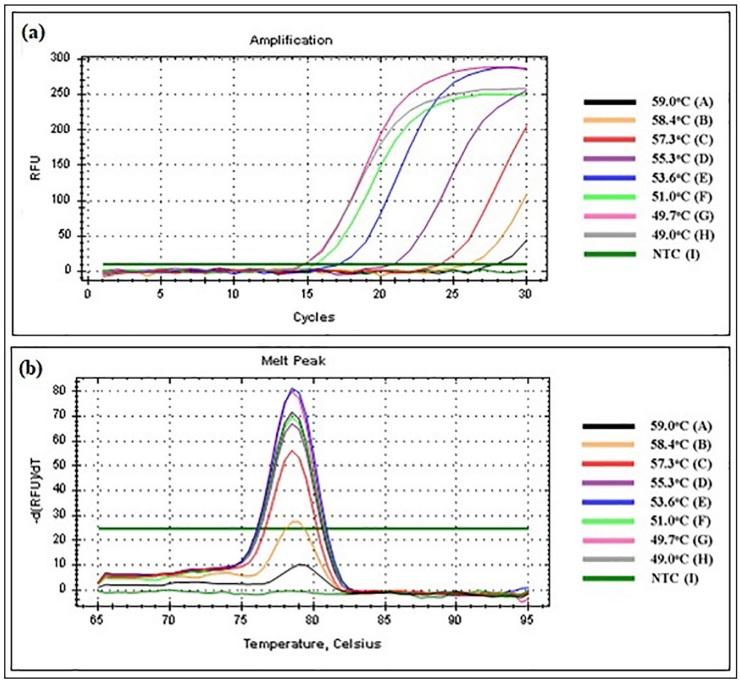

Fig. 1. The optimization results of primer annealing temperature of ND2 set 4 on Bengal rat DNA isolate. (a) Amplification curve at different 8 temperature levels; (b) melt-curve analysis.

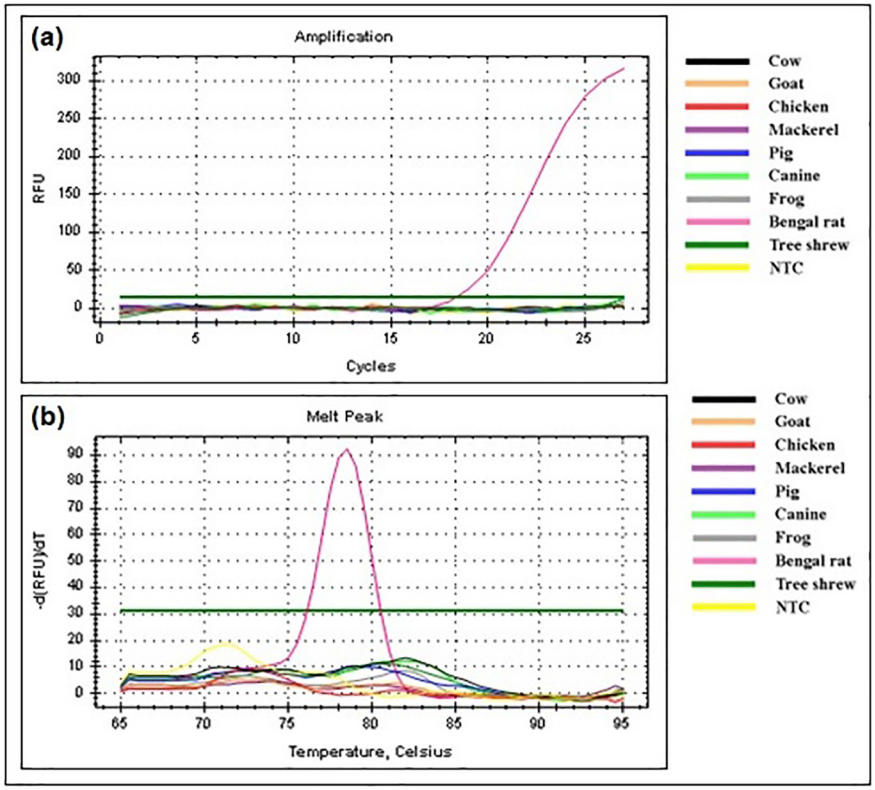

Fig. 2. The specificity test results for ND2 set 4 primers. (a) Amplification curve. (b) MCA.

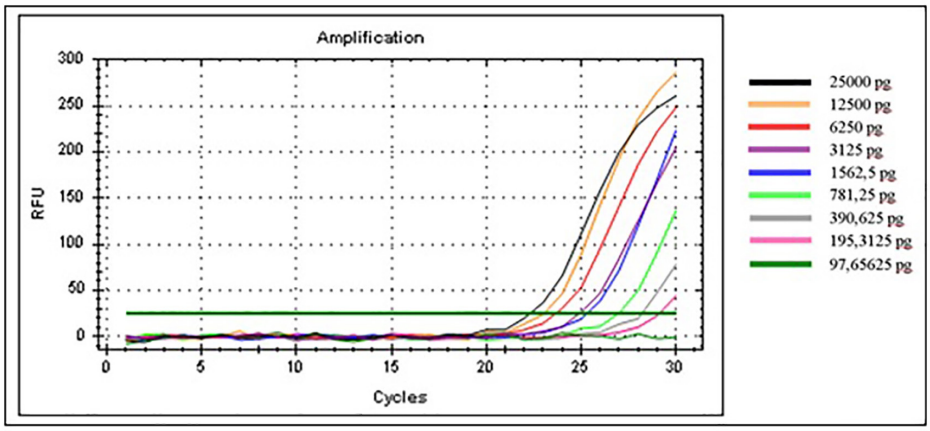

Fig. 3. The senitivity test result of Bengal rat DNA at different levels of DNA using ND2 set 4 primers.

non-target species (cows, goat, chickens, mackerel, canine, pigs, frogs, and tree shrew) along with negative control or NTC at an optimum Ta previously obtained (53.6°C) for 27 cycles. The amplification curve and melt curve are parameters considered to determine the specificity of the RT-PCR reaction. The amplification curve data (Fig. 2a) showed that the ND2 set 4 primers only amplified the DNA template of the Bengal rat with no amplification observed in DNA isolates from others and negative control (NTC). Furthermore, the specificity of the RT-PCR reaction was confirmed using melt curve analysis (MCA) applied for the analysis whether the RT-PCR reaction is capable of producing a single product. Figure 2b shows that MCA data only produces a single peak. Therefore, it can be concluded

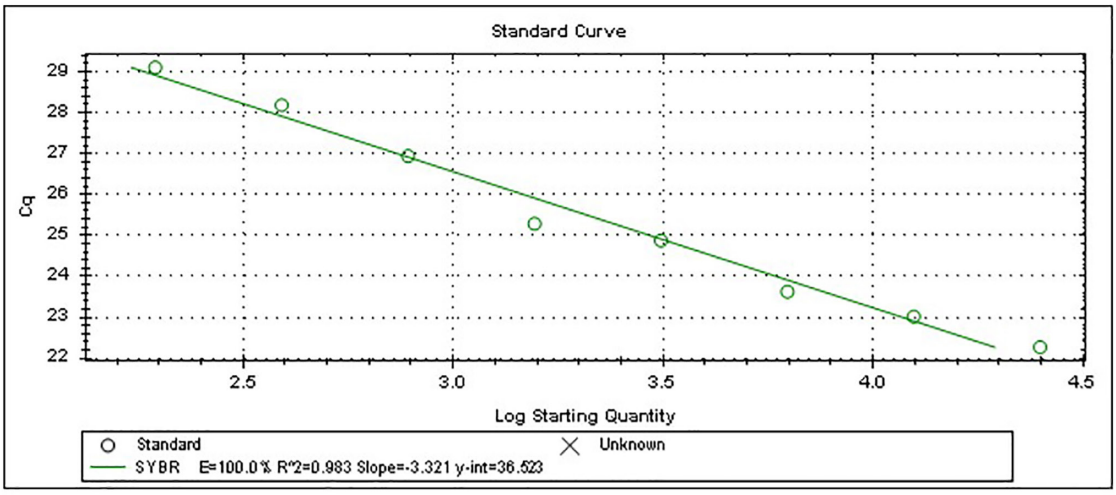

Fig. 4. The calibration curve correlating the different levels of log concentration of DNA (*x*-axis) and Cq-values (*y*-axis) using ND2 set 4 primers.

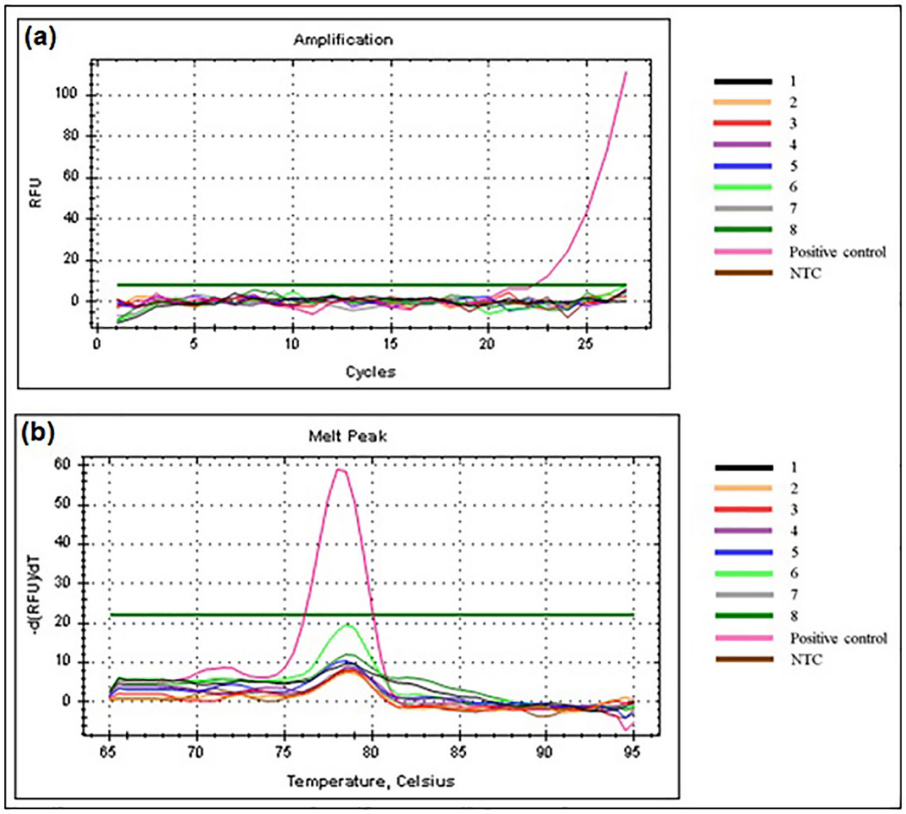

Fig. 5. Application of validated RT-PCR method for identification marketed beef meatball samples. (a) The amplification curve of meatball samples using ND2 set 4 primers. (b) The melt-curve analysis.

that the ND2 set 4 primers had good specificity for Bengal rat DNA under RT-PCR conditions.

The sensitivity test of the real-time PCR method was evaluated by determining the LoD value of DNA isolated from Bengal RM. LoD is expressed as the lowest quantity of target DNA which still can be detected and amplified. The LoD value evaluation based on the amplification of isolate DNA of Bengal

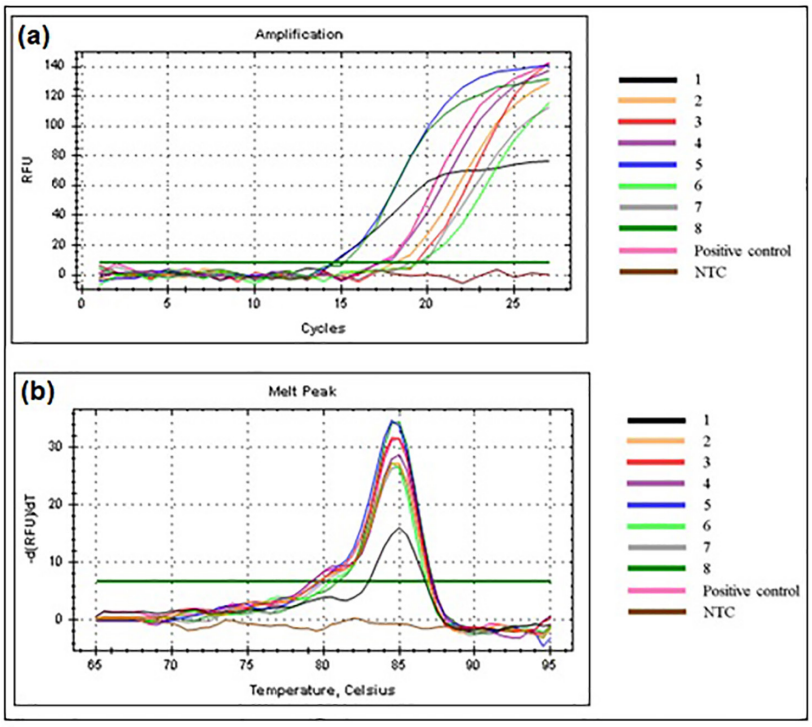

Fig. 6. Analysis of meatball samples using bovine DNA-specific primer to confirm the presence of bovine DNA in the meatball samples. (a) Amplification curve. (b) MCA.

rat with 2-fold serial dilution at a concentration of 25,000, 12,500, 6,250, 3,125, 1,562.50, 781.25, 390.63, 195.31, and 97.66 pg using an optimum annealing temperature. The amplification curve (Fig. 3) shows that at a concentration of 195.31 pg, Bengal rat DNA was still amplified with a Ct value of 29.10, while at a concentration of 97.66 pg, the amplification curve was not detected. As a result, the LoD value for Bengal rat DNA was 195.31 pg. The resulting amplification was then transformed into a standard curve by plotting the log value of DNA concentration (*x*-axis) and the Ct value (*y*-axis) for evaluating the amplification efficiency value (E). As shown in Figure 4, the E-value obtained from this curve was 100%. Although the efficiency value reflects the ideal amplification reaction, the linearity value (R^2) indicates that the PCR reaction conditions did not occur ideally. These R^2 and *E* values meet the recommended testing criteria for the simplex qualitative RT-PCR method ($R^2 \geq 0.98$ and *E* 80%–120%) (Broeders *et al*., 2014). The efficiency value was calculated based on (Rojas *et al*., 2010),

$E = (10^{(-1/slope)} - 1) \times 100$

$\%E = (E - 1) \times 100\%$

The repeatability test was used to evaluate the precision of the developed method. The evaluation of repeatability was performed by calculating the RSD of the Ct value of the target DNA response from six replicates. The repeatability test was using a concentration of 6,250 pg. Ct values were in the range of 23.48–24.52, with an RSD value of 1.80%. According to the guidelines of qualitative RT-PCR analysis, the acceptance criteria for RSD is ≤ 25% (Widyasari *et al*., 2015) and the Codex Allimentarius Commission required that the PCR method be considered precise if maximum RSD values of 25% were obtained. Therefore, real-time PCR using a designed primer was precise enough and the random error occurring during analysis can be negligible. The results reveal that the CV value achieved met the acceptance criteria (Comission, 2010). The results reveal that the CV value achieved met the acceptance criteria. Low RSD values indicated that the developed method was precise and applicable to be used for RT-PCR analysis.

Eight marketed beef meatball samples were purchased from local food stalls in Yogyakarta, Indonesia. The analysis process for marketed beef meatball samples begins with the DNA isolation stage using the same method. The successful analysis of meat species identification using real-time PCR depends on the yield and quality of DNA extracted from fresh meat and meatballs. DNA may be heterogeneous in composition due to processing. DNA must comply with minimum requirements about (1) integrity, which determines the fraction of DNA that PCR can amplify, (2) yield in which DNA has to be in sufficient amount for analysis, and (3) purity, which plays a crucial role in

DNA analysis because the presence of carbohydrates, proteins, and other metabolites can interfere during real-time PCR reactions which can be assessed based on the size distribution of fragmented DNA (Cravero *et al*., 2019). All meatball samples met the purity index requirement, in which A_{260}/A_{280} was within the range of 1.8–2.0. However, if samples have A_{260}/A_{280} above 2.0, indicating the presence of RNA, and some samples with A_{260}/A_{280} < 1.8 indicate the presence of protein and carbohydrate in extracted DNA, as shown in another study have A260/A280 lower than 1.8 and higher than 2.0 (Rohman *et al*., 2020). The DNA isolates of the market samples were tested using two pairs of primers, namely the ND2 set 4 primers which had been previously designed, and a bovine DNA-specific primer to confirm the presence of bovine DNA in the meatball samples. The validated RT-PCR using ND2 set 4 primers only amplified the DNA template of the Bengal rat (positive control) with a Ct value of 22.30, while the eight DNA isolates from meatball samples and negative control (NTC) did not show any amplification response (Fig. 5a). Furthermore, MCA (Fig. 5b) shows that only a single peak is formed in the positive control, while the eight meatball samples and NTC have a melting peak below the threshold limit. Using bovine DNA-specific primer, eight DNA isolates from meatball samples and positive control showed a similar amplification response to the positive control. On the other hand, the data results also show that there is no amplification response in the negative control (Fig. 6a). Furthermore, the MCA of eight market samples, as well as positive control, gave a uniform MCA profile with a Tm value of 85°C (Fig. 6b). Therefore, it can be concluded that all of the meatball samples did not contain Bengal rat DNA and contained bovine DNA.

Conclusion

The primer specifically designed to target the ND2 gene in the mt-DNA of Bengal rats demonstrated excellent specificity under the experimental circumstances employed (annealing temperature of 53.6°C for 27 cycles) as compared to eight other species, namely cows, goats, chickens, mackerel, canines, pigs, frogs, and tree shrews. The RT-PCR method that was developed has a LoD value of 195.31 picograms, a coefficient of variation (CV) value for the amplification response of 1.8%, a linearity value of 0.983, and an amplification efficiency (E) value of 100%. The findings indicated that the RT-PCR technology that was created did not identify any traces of Bengal rat DNA in 8 of the marketed beef meatball samples. The implementation of the RT-PCR technique resulted in a reliable, accurate, and targeted detection method for identifying the Bengal rat. The method developed successfully fulfills the validation testing criteria for RT-PCR and can be employed as a halal verification technique for detecting the presence of Bengal rat meat in beef meatballs.

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Authors' contributions

AR: Planned the study and drafted and revised the manuscript. HHN, HW, MH, MDL, and DL: Performed the extraction of DNA and analyzed the data. All authors have read, reviewed, and approved the final manuscript.

Conflict of interest

The authors state no conflict of interest.

Data availability

All data supporting the findings of this study are available within the manuscript and no additional data sources are required.

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