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The use of real-time polymerase chain reaction for detection of raw rat meat (*Rattus norvegicus*) with species-specific primer for halal authentication

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Abstract

Rat meat (RM) can be obtained freely from farmers and can be used as a potential adulterant in meat-based food products. The presence of RM in any food products is prohibited by the Muslim community and is considered non-halal meat. Therefore, an analytical method capable of analysing RM specifically is very urgent. In this study, a real-time polymerase chain reaction using a species-specific primer targeting NADH dehydrogenase subunit 6 (ND6) is applied to analyze RM. DNA extraction was carried out using FavorPrep^{TM} Tissue Genomic DNA Extraction Mini Kit, and the extracted DNA was subjected to purity index using NANO-Quant SPARK TECAN. The annealing temperature (Ta) used for PCR analysis was optimized to get the best Ta capable of providing the optimum amplification reaction. Furthermore, some performance characteristics were evaluated for Real-Time PCR, including sensitivity, efficiency and repeatability. The results exhibited that the designed primer is specific to rat DNA extracted toward other DNAs from meats typically used in food products. For quantitative evaluation, the limit of detection found was 0.39 ng with an efficiency of amplification (E) of 94.4% with a coefficient of determination (R^2) of 0.986 for the relationship between log concentrations of DNA and cycle threshold (Ct) values. The repeatability assay was acceptable with an RSD value ≤ 25%. The developed method was reliable, provided high sensitivity for the analysis of RM. Therefore, this method is suitable to be used as a standard method for halal authentication analysis.

1. Introduction

Meat-based food products such as meatballs and sausages are a favorite food among Indonesian people because they are protein sources needed for human development. Beef meatballs and sausages are the meat-based products typically found in the markets (Martuscelli *et al*., 2020). Today, the price of beef (halal meat) is higher than other halal meats such as chicken and non-halal meats like pork and wild boar meat. Unethical producers are attracted to blending or substituting halal meat with non-halal meats such as pork, canine meat, wild boar meat, and rat meat to get economic profits (Ahda *et al*., 2020). The presence of non-halal meats in food products is a severe problem because Muslims are not allowed to consume products containing non-halal components (Mursyidi, 2013). In many countries, adulterating meat and meat-based products is a severe problem in the food industry sector. This adulteration practice can disturb not only the economy of a particular country but also reduce consumer confidence in those products. In addition, it can aggravate some allergenic reactions in certain people (Denyingyhot *et al*., 2022). The adulteration of non-halal meats is a concern not only for consumers but also for producers and government or regulators.

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Indonesia has implemented Indonesian Act No. 33 (2014) on Halal Products Assurance, stipulating that all halal products entered, distributed, and commercialized in Indonesia must be certified halal (Adiarni and Fortunella, 2018). This implies that the products must be free from non-halal components; therefore, the availability of analytical methods capable of detecting non-halal meats is very urgent (Rohman, Pebriyanti, Sismindari *et al*., 2020). Several analytical methods have been developed and applied for rapid detection of non-halal meats such as portable FTIR spectroscopy in combination with multivariate analysis for differentiation of pork and others (Dashti *et al*., 2022), Raman spectroscopy (Ghazali and Tukiran, 2021), different scanning calorimetry (DSC) through analysis of thermal properties in lipids extracted from non-halal meats (Rohman and Che Man, 2012), liquid chromatography-Orbitrap high resolution mass spectrometry through metabolomics and proteomics studies for analysis of pork adulteration (Windarsih *et al*., 2022), enzyme-linked immunosorbent assay (ELISA) through analysis of protein in non-halal meats (Zvereva *et al*., 2015), gas-chromatography-mass spectrometry (GC-MS) through volatilomics (analysis of all volatile compounds) of non-halal meats (Pranata *et al*., 2021), electronic nose (e-nose) or fast gas chromatography for analysis of volatile compounds (Nurjuliana *et al*., 2011), electrochemical biosensors (Flauzino *et al*., 2022) and protein-based laser -induced breakdown spectroscopy for the identification of pork and others in the fermented sausage and salami products (Sezer *et al*., 2022). Some methods lack specificity (e-nose, DSC) and involve sophisticated instruments (GC-MS, LC-MS); therefore, analytical methods based on DNA were continuously developed and validated for the analysis of non-halal meats.

DNA-based methods using polymerase chain reaction (PCR) are the method of choice or gold method for analysis of DNA extracted from meat and meat-based food products (Salihah *et al*., 2016). PCR, along with its development, such as PCR-hybridization, multiplex PCR (Ali *et al*., 2015), and real-time PCR offered a specific method for detecting DNA extracted from non-halal meats (Baptista *et al*., 2021). Specifically, real-time PCR provides a rapid and inexpensive method for detecting and quantifying the targeted DNA segments in meat-based food samples, helping determine either accidental or intentional adulteration practices of foods. Real-time addressed some limitations met in conventional PCR regarding operational time, sensitivity, range of analytes, multiplexing ability, and point-of-care applications. Real -time using species-specific primers are reported to be successfully employed for the analysis of DNAs from non-halal meats in food products such as pork (Aida *et al*., 2005), wild boar meat (Aina *et al*., 2020), and canine meat (Kurniasih *et al*., 2019). In this study, real-time PCR using species-specific primer targeting NADH dehydrogenase subunit 6 (ND6) is applied to analyze rat meat for halal authentication analysis.

2. Materials and methods

2.1 Materials

The main raw materials of rat meats (*Rattus norvegicus*) were obtained from Godean, Sleman, and Yogyakarta; beef (*Bos taurus*), chicken (*Gallus gallus*), pork (*Sus scrofa domesticus*), and dog meat (*Canis lupus familiaris*) were obtained from Yogyakarta, Indonesia.

2.2 Extraction of DNA

DNA was extracted from 25 mg of the raw samples using FavorPrepTM Tissue Genomic DNA extraction mini kit (FAVORGEN, Austria), following the manufacturer's instructions. Firstly, the sample was ground with a micro pestle in a 1.5 mL micro-centrifuge tube to make a pulp, followed by adding 200 µL FATG1 Buffer and mixing well with a micro pestle or pipette tip. Furthermore, add 20 µL Proteinase K to the sample mixture, mix thoroughly by vortexing, then incubate at 60.0°C until the tissue is lysed completely (1~3 hrs). Vortex occasionally during incubation. After incubation, add 200 µL FATG2 Buffer to the sample mixture, mix thoroughly by pulse-vortexing and incubate at 70.0°C for 10 mins. Then Add 200 µL ethanol (96-100%) to the sample mixture. Mix thoroughly by pulse-vortexing. Place a FATG mini-column in a collection tube. Transfer the mixture (including any precipitate) to the FATG mini -column. Centrifuge at full speed (~18,000×*g*) for 1 min, then place the FATG mini-column into a new collection tube. Add 400 µL W1 Buffer to the FATG mini-column. Centrifuge at full speed for 1 min, then discard flow-through. Add 750 µL wash buffer to the FATG mini-column. Centrifuge at full speed for 1 min, discard flow-through, and the last step adds 100 µL of preheated elution buffer, centrifuge at full speed for 2 mins to elute DNA (Marie, 1996).

2.3 Designing of primers

One set of primers targeting ND6 was designed using the software National Center for Biotechnology Information (NCBI, Maryland, USA) in silico using accession number MW209726.1 for DNA ND6 rat meat. Primer was subjected to BLAST (Basic Local Alignment Search Tool) from the NCBI website.

2.4 Real-time PCR analysis

Amplification was performed in a final volume of 10 µL, 5 µL SensiFAST™ SYBR®, 0.5 µL of each primer, and 3.5 µL of free nuclease water. The amplification was

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performed with a real-time PCR using PCR CFX96® (Biorad, USA). The thermal cycler protocol was as follows: pre-denaturation at 95°C for 2 mins, followed by 30 cycles of denaturation at 95°C for 10 s, annealing at 59°C (the optimum annealing temperature) for 15 s, and extension at 72°C for 10 s (Rohman *et al*., 2021).

2.5 Validation of real-time PCR using designed specifically primer

The primer design was further validated by determining several parameters: specificity, efficiency, sensitivity, and repeatability (Safdar *et al*., 2014; Perestam *et al*., 2017). The specificity test of primer ND6 was evaluated by amplifying DNA extracted from several types of meat (rat, beef, chicken, pork, and dog). The sensitivity of real-time PCR using primer ND6 was expressed by the limit of detection (LoD). LoD was evaluated by making a dilution series of DNA extracted from rat meat at a concentration of 6250 pg, 3125 pg, 1562.5 pg, 781.25 pg, and 390.63 pg. LoD value was the lowest amount of DNA that could be amplified with reproducible cycle threshold (Ct) value or cycle of quantification (Cq) value. The repeatability assay for precision evaluation was performed by replicating these dilution series in five replicates (Murugaiah *et al*., 2009).

3. Results and discussion

Real-time PCR is one of the DNA-based methods. In this research, real-time PCR was performed on DNA extracted from muscle tissues of each target species to verify the specificity of the designed primers. DNA isolation was carried out by FavorPrep^{TM} Tissue Genomic DNA Extraction Mini Kit. The DNA isolation method was carried out in various steps, including cell membrane lysis, DNA extraction using organic solvents, refining, precipitating, and concentration. The isolated DNAs from raw meat were subjected to spectroscopic measurement using Spark® Multimode Microplate Reader (Tecan, Switzerland) to determine DNA concentrations and purity. The result ratios of extracted DNA were between 1.7 and 2, indicating a high DNA quality in all specimens, as shown in Table 1 (Rohman, Pebriyanti, Sismindari *et al*., 2020). A specific primer combined with real-time PCR is essential to develop a robust and reliable method for analyzing rat meat DNA. The designed primers were targeted on short DNA amplicon (150 bp). Table 2 shows the primer of *Rattus norvegicus* (forward CAAGTCTCCGGGTACTCCTC and reverse GATTGTTAGTGGATGTATTGGGTGC). Some advantages of short amplicons are capable of amplifying DNA targets more efficiently and resulting in better recovery of targets (Rohman, Windarsih, Erwanto *et al*., 2020). The primer targeting the ND6 was designed using the NCBI software to identify and quantify rat meat DNA using real-time PCR. This gene was chosen because it revealed an appropriate degree of variability of intra- and inter-species. This gene also offers a high number of copies per cell, which significantly increases the sensitivity of the real-time assay and contributes to the survival of a few DNA copies when tissue has been subjected to extreme processing conditions, such as sterilization and boiling processes (Rohman *et al*., 2021). The selected primer having G or C bases in the last five positions of the 3' end less than three can increase the specific binding at the 3' (Widyasari *et al*., 2015).

Table 1. The DNA concentration and purity of DNAs extracted from raw meats.

Meat types	The concentration of DNA (ng/µL)	Purity index (A_{260}/A_{280})
Rat meat	167.01	1.95
Beef	91.45	2.00
Chicken meat	257.61	2.08
Pork	89.13	1.96
Dog meat	135.56	1.94

A real-time PCR instrument using primer ND6 was validated by determining some parameters, namely primer specificity, efficiency, sensitivity, and repeatability (Bustin *et al*., 2009). For the specificity test, DNA from rat meat (*Rattus norvegicus*) and DNA from beef (*Bos taurus*), chicken (*Gallus gallus*), pork (*Sus scrofa domesticus*), and dog (*Canis lupus familiaris*) were extracted. The optimization of annealing temperature (Ta) used during PCR analysis was initially performed. Figure 1 shows optimum Ta was temperature capable of amplifying the DNA target specifically. Ta is generally 3–5°C below the melting temperature;

Table 2. The primer of *Rattus norvegicus* (Forward and Reverse) and its characteristic was selected from the primer designing process using NCBI – Primer-BLAST software from the NCBI website.

Primer	Length	Tm	GC%	Self-Complementarity	Self-3' Complementary
Forward:	20	58.9	60.00	6	1
CAAGTCTCCGGGTACTCCTC	25	60.22	60.22	2	2
Reverse: GATTGTTAGTGGATGTATTGGGTGC					
Product length: 150					
(ND6) Genbank MW209726.1					
https://www.ncbi.nlm.nih.gov/nuccore/MW209726.1					

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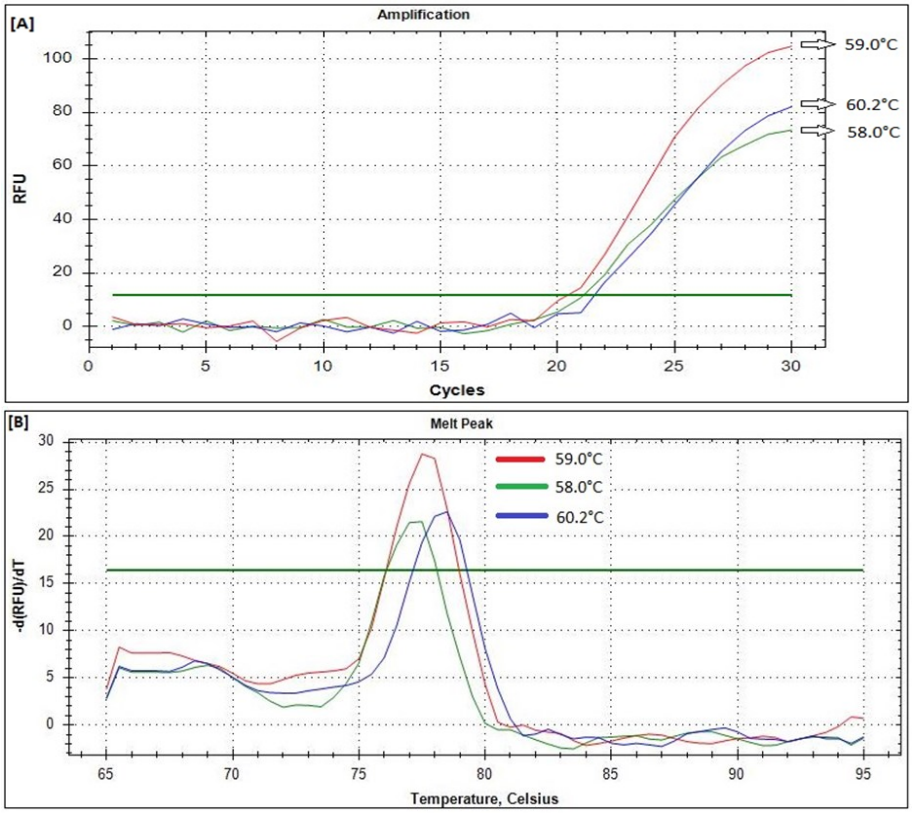

Figure 1. Optimizing the annealing temperature of ND6 primers amplifies the DNA target extracted from raw rat meat (*Rattus norvegicus*); the optimum annealing temperature was 59.0°C. [A] The amplification curves and [B] Melting peak.

therefore, Ta was optimized at 58–60°C. Finally, a temperature of 59.0°C was chosen as the optimum Ta with a quantification cycle (Cq) of 20.44. The result revealed that primer ND6 could specifically amplify rat meat (RM) DNA and did not amplify other DNA used (Figure 2).

The sensitivity of the ND6 primer was evaluated by determining the limit of detection (LoD). LoD was evaluated by preparing a dynamic range of serial DNA concentration covering 6250 pg, 3125 pg, 1562.5 pg, 781.25 pg, and 390.63 pg) of rat meat DNA (x-axis) and the value of quantification cycle (Cq) (y-axis). The results (Figure 3) showed that the absolute LoD for rat meat DNA was 390.63 pg (0.39 ng). The standard curve showed a coefficient of determination (R^2) of 0.986, slope -3.463, and y-intercept 34.412. These values meet the criteria of the linearity curve, while the amplification efficiency is at 94.4%, following the requirements in Bio -Rad (2006), namely 90-110%.

The repeatability test evaluated the precision of Real -Time PCR. Repeatability tests were performed on raw rat meat (Figure 4) with a concentration of 1562.5 pg (1.56 ng) to confirm the consistency of the Real-Time PCR result. Template DNA was amplified using an ND6 primer with five replicates, and a relative standard deviation (RSD) value of 0.460% was obtained. Real-time PCR analysis was considered precise if the RSD value was <25% and required that the coefficient of variation (CV) is less than 25% (European Network of GMO Testing (ENGL), 2008) (Rohman *et al*., 2021). The repeatability test demonstrated that the amplification occurs in the sample (Table 3) with a CV of raw rat meat is 1.793%. The developed Real-Time PCR assay could be used to analyze raw rat meat for halal authentication.

Table 3. Repeatability test at concentration 1.56 ng for raw rat meat (*Rattus norvegicus*) DNA.

Rat DNA Concentration	Cq	Cq Average	SD	CV
1.56 ng	25.55			
1.56 ng	25.75			
1.56 ng	26.15	25.654	0.46	1.793
1.56 ng	25.89			
1.56 ng	24.93			

4. Conclusion

Real-time PCR using a specific primer of ND6 at an annealing temperature of 59°C was successfully used to identify and quantify raw rat meat. The validation results revealed that real-time PCR was fit for purposes of the identification of rat meat DNA. The developed PCR method can be used in the identification of rat meat in food products to support the development of standard methods in halal analysis.

Conflict of interest

The authors declare no conflict of interest.

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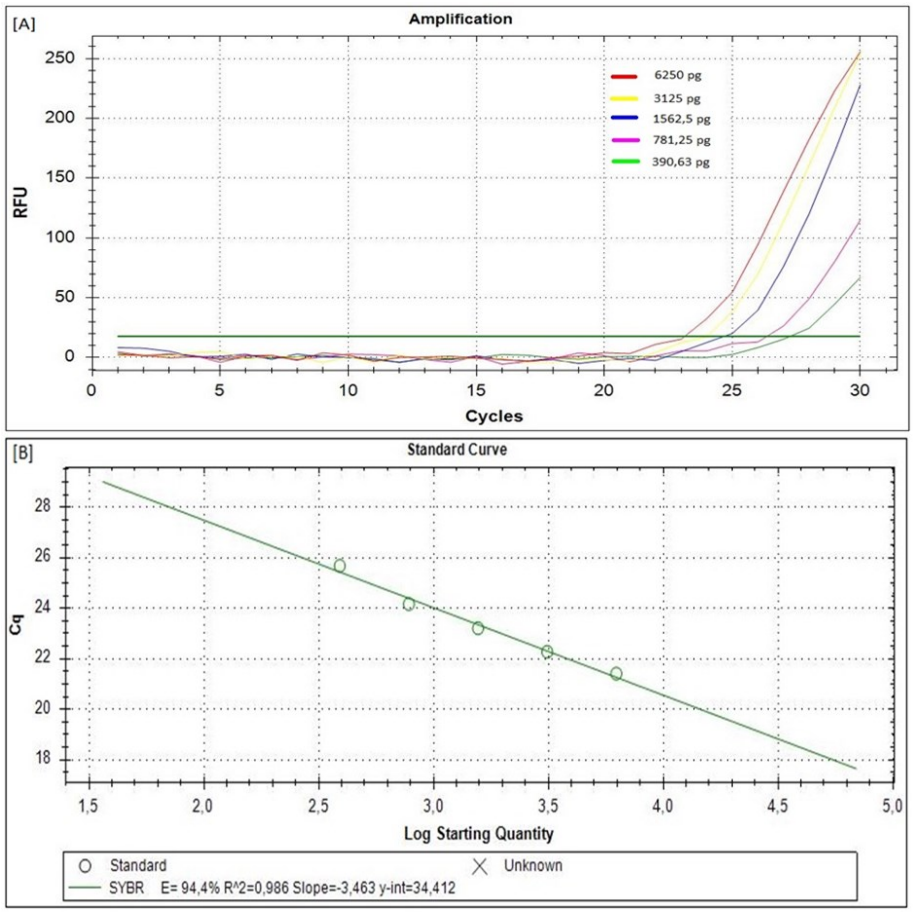

Figure 3. Real-time PCR amplification using ND6 primers toward rat DNAs diluted at five different concentrations. [A] Amplification curves, [B] Calibration curves.

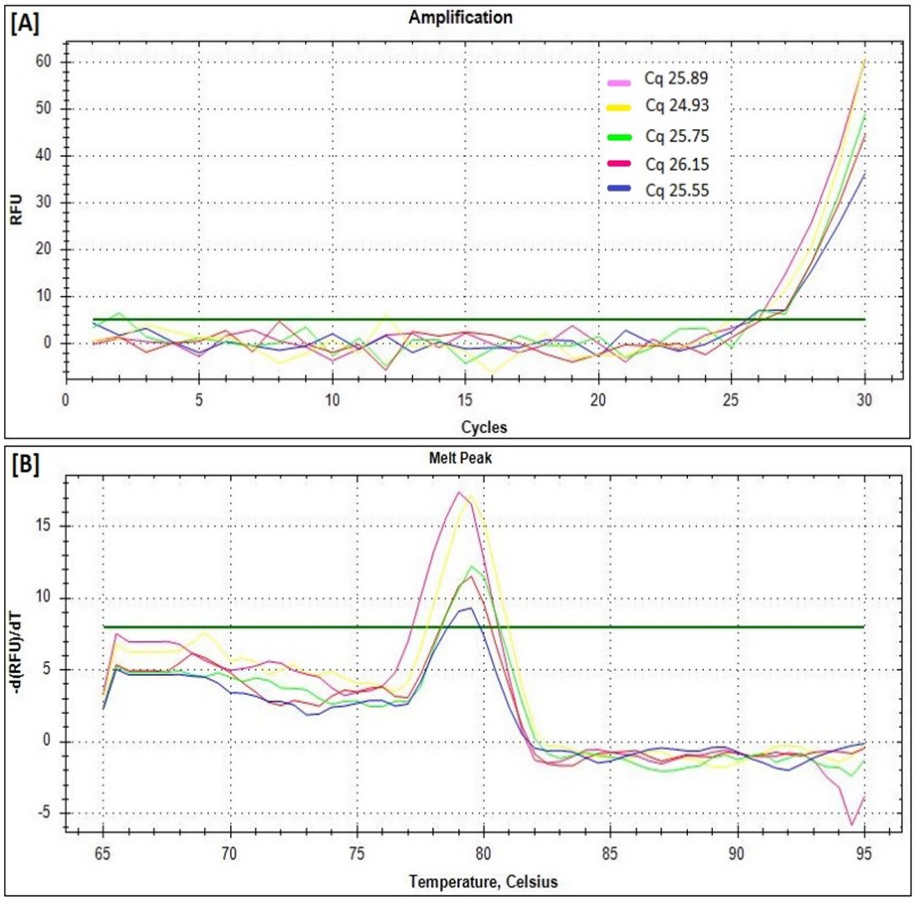

Figure 4. A repeatability test at template DNA at a concentration of 1562.5 pg (1.56 ng) was amplified using ND6 primers with five replicates. [A] Amplification curves, [B] Melting curve analysis.

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