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Application of real-time PCR for analysis canine meat (*Canis lupus familiaris*) in goat's satay for halal authentication study

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ABSTRACT

Background: Canine meat (CM) is one of the non-halal meats prohibited for consumption by the Muslim community. Due to its low prices compared with beef, CM is typically used as meat adulterants in halal food-based products such as Satay and meatballs to get economic profits.

Aim: The objective of this study was to design a novel species-specific primer in combination with real-time polymerase chain reaction for analysis of Canine's DNA for halal authentication analysis.

Methods: A Primer targeting the D-loop region of mitochondrial DNA was designed and subjected to a validation procedure by assessing some performance characteristics including specificity, amplification efficiency (E), sensitivity, repeatability, and linearity describing the correlation between the concentration of Canine's DNA (*x*-axis) and quantification cycle (Cq) in *y*-axis. The designed primer was specific over other meat DNAs applying the annealing temperature (Tm) of 57.8°C.

Results: The Real-Time Polymerase Chain Reaction (RT-PCR) method produced an acceptable amplification efficiency (E) of 109.7% with the coefficient of determination (R^2) for the correlation between Cq and log DNA concentration of 0.999. The sensitivity of the developed method provides a limit of detection (LoD) value of 31.25 pg/µl. The precision of the analytical method is acceptable with a relative standard deviation value of 2%. The method with the designed D-loop primer was successfully applied for the detection and quantification of Canine's DNA in food products. There are no amplification profiles for Canine DNA in marketed goat's satay products.

Conclusion: RT-PCR combined with a novel primer targeting D-loop provides a specific and accurate analytical tool for the identification of CM for halal authentication studies.

Keywords: D-loop primer, Real time-PCR, Canine meat, Goat's satay.

Introduction

In recent years, the substitution of halal meats having high prices in the market such as beef with non-halal meats with lower prices such as pork, rat meat, and dog meat has been reported in some scientific literature (Pebriana *et al.*, 2017). Erwanto *et al.* (2014) reported that pork contamination was found at 9 of 20 meatball stalls in Yogyakarta regions. This action is motivated by economic reasons in which halal meats are more expensive than non-halal meats, or even non-halal meats can be freely available such as canine meats (CMs) (Kurniasih *et al.*, 2019). According to Indonesia's National Strategic Food Price Information Center, the price of beef is 130 k/kg, while the price of CMs at the slaughterhouse starts at 30 k–50 k/kg. One of the favorite meat-based food products in Indonesia is Satay, and Satay's name is typically derived from meat sources such as Beef's Satay, Chicken's Satay, and Goat's satay. As a popular grilled food delicacy, Satay is also popular in Southeast Asian regions including

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Malaysia, Thailand, and Singapore. Satay is similar to shish kebab which is popular in Turkey and in Middle Eastern countries (Jinap *et al.*, 2013). Among these Satay, goat's Satay is the most consumed by Indonesian people (Irnanda *et al.*, 2012), as a consequence, goat's meat is the potential to be adulterated or substituted with non-halal meats. For this reason, the availability of analytical methods capable of confirming the presence of non-halal meats is very urgent.

Identification of meat types in meat-based food products is very urgent, especially for the identification of non-halal meats. Indonesia has implemented the Indonesian Act No. 33 the year 2014 on Halal Products Assurance stating that all products including meat-based products distributed and sold in Indonesia must be halal-certified, and the products suspected to contain non-halal meats must be subjected to testing in an accredited laboratory according to ISO/IEC 17025: 2017 (Malik *et al.*, 2016). Several analytical methods have been proposed and used for the identification, detection, and confirmation of non-halal meats in food products, mainly based on molecular spectroscopic methods including Fourier Transform Infrared (FTIR) spectroscopic (Kuswandi *et al.*, 2015), Nuclear Magnetic Resonance (NMR) spectroscopy (Fadzillah *et al.*, 2017) and Raman spectroscopy in combination with multivariate data analysis (Mortas *et al.*, 2022) and chromatographic-based methods including gas chromatography hyphenated with mass spectrometer or GC-MS (Indrasti *et al.*, 2010) and liquid chromatography hyphenated with mass spectrometer or LC-MS/MS (Abbas *et al.*, 2020). In addition, some screening methods for rapid detection of non-halal meats are also proposed such as electronic nose (Nurjuliana *et al.*, 2011), electronic tongue (Tian *et al.*, 2019), and differential scanning calorimetry or DSC (Bertram *et al.*, 2006). All these methods need advanced data treatment which involves big data analysis, therefore some methods based on specific markers such as protein and DNA-based methods are widely used.

DNA-based methods using polymerase chain reaction are currently taken into account as a method of choice or gold methods for identification and confirmation of non-halal meats in food products due to their specificity and sensitivity. Numerous PCR methods have been reported for the identification of non-halal meats in food products including real-time PCR and conventional PCR using species-specific primers (SSPs), singleplex or multiplex PCR, random amplified polymorphic DNA, and restriction fragment length polymorphisms as reviewed by some authors (Salihah *et al.*, 2016; Rohman *et al.*, 2020a,b; Muflihah *et al.*, 2023). Real-time PCR and conventional PCR using SSP targeting on mitochondrial, cyt-b, and d-loop and other PCR techniques have been successfully applied for analysis of non-halal meat-based products such as analysis of pork in meatball (Erwanto *et al.*, 2012), analysis of pork in abon (Rahmawati *et al.*, 2016), analysis of dog meat in meatball (Ali *et al.*, 2015), and analysis of rat meat in meatball (Cahyadi *et al.*, 2020).

D-loop has good specificity in identifying raw materials in food products which has been processed through cooking and degradation (Kusnadi *et al.*, 2020). Many studies related to food identification using D-loop primers have been conducted, such as analysis of pork in sausages (Maulani *et al.*, 2020), analysis of pork gelatin in capsule shells (Sudjadi *et al.*, 2016), and analysis of monkey meat contamination in beef meatballs (Kurniawati *et al.*, 2024). The use of D-loops in detecting foods is considered to have a high success rate, so it has a high opportunity to be used in halal detection methods with various PCR-based methods (Sosiawan *et al.*, 2020). From literature searching, the identification of non-halal meats in Satay is very limited. Therefore, the objective of this study was to design SSP followed by real-time PCR analysis for the identification of CM in Goat's Satay. In addition, the developed real-time PCR using SSP targeting on D-loop mitochondria was validated by determining some performance characteristics which included primer specificity, sensitivity, extraction efficiency, reproducibility, and application of real-time PCR for analysis of Satay commercially available in the market.

Materials and Methods

Experimental details and treatment

CM and rat meat were obtained from markets and farmers. The other types of meats namely goat meat, mackerel, pork, chicken, beef, and frog meat were purchased from traditional markets in Yogyakarta Province, Indonesia. Ten commercial goat's satay samples were collected from Yogyakarta, Indonesia. Some sets of primers for real-time PCR analysis were purchased from Genetika Science (Indonesia).

Primer designing

A set of primers targeting D-loop was designed using software from Integrated DNA Technologies (IDT, California, USA). DNA sequence of *Canis lupus familiaris* mitochondrion DNA complete genome (MW549038.1) was obtained from NCBI (http://www.ncbi.nlm.nih.gov). A set of primers was selected from five primers candidates from IDT after *in silico* specificity test using BLASTn (Basic Local Alignment Search Tool Nucleotide) from the NCBI website by comparing canine species with goat, rat, mackerel, pig, frog, cow, chicken, cow, and tree shrew. The selection of primer also considers the presence of the predicted secondary structure of oligonucleotides using OligoAnalyser Tool from IDT Software. The selected primer for further analysis and validation is shown in Table 1.

DNA isolation

The isolation of DNAs from raw meat and Satay products were isolated using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen, Taiwan). The procedure

of isolation followed the manufacture's instruction with several stages, including cell lysis using buffer lysis and proteinase-K, binding DNA with a silica column matrix, washing DNA from a contaminant, and eluting DNA from the silica column.

Analysis using real-time PCR

Real-time PCR amplification was carried out using PCR CFX96 (Biorad, USA) with a total volume of 10 µl containing 5 µl of 2x SensiFAST SYBR® No-Rox Mix (Meridian Bioscience, USA), 0.4 µl of each forward and reverse primers (10 µM), 1 µl template DNA with concentration 25 ng/ µl and 3.2 µl nuclease-free water. The reaction was carried out in 25 amplification cycles on the program with cycling conditions used was as follows: pre-denaturation at 95ºC for 3 minutes, denaturation at 95ºC for 5 seconds, annealing step at 57.8ºC for 10 seconds, and elongation step at 72ºC for 20 seconds.

Validation of real-time PCR methods

Real-time PCR using an SSP primer previously designed was subjected to validation by determining several parameters, including specificity, efficiency, sensitivity, and repeatability as in Codex Alimentarius Commission (CAC) (Aina *et al.*, 2019). The validated methods were then employed for the identification of canine DNA in commercial goat's Satays by amplifying real-time PCR methods using primer D-loop 38 for analysis of 10 commercial goat's satay samples with canine's DNA as positive control and no template control (NTC) as negative control.

Data analysis

The isolated DNAs were subjected to the purity assessment and the concentration of DNAs was quantified using Nano Quant Spark Tecan (Switzerland) at Wavelengths of 260 and 280 nm. Selection of the optimum annealing temperature was carried out by looking at the increase of the curve and the cycle threshold (Ct) value of the amplification curve. The specificity test of primer D-loop was evaluated by amplifying DNAs isolated from several types of meats (canine, goat, cow, pig, mackerel, Bengal rat, tree shrew, chicken, and frog) and NTC. The sensitivity of real-time PCR using D-loop was carried out by determining the limit of detection (LOD), as the lowest quantity or concentration of DNA that still can be reliably detected and amplified with reproducible Ct using real-time PCR. The LOD value was evaluated using several dilutions of the isolated DNAs from raw CM and then amplified with a designed primer. The amplification efficiency (*E*-value), slopes, and coefficient of determination (R^2) were calculated using the software Bio-Rad CFX manager. Efficiently was aimed to know the capability of the real-time PCR method in amplifying the DNA targets and it was determined from standard curve plotting Ct values (*y*-axis) versus log concentration of DNA (*x*-axis). The repeatability of the validated method was carried out using eight replicates.

Results and Discussions

In this study, the SSP was specifically designed using NCBI software for the identification of CM in processed foods, especially in Satay products using real-time PCR methods. The primer was designed to targeting D-loop fragment of mitochondrial DNA (mtDNA) *C. familiaris*. Some advantages of using D-loop fragment are that it has the highest levels of polymorphism in mtDNA and has good specificity in the identification of food processing products (Liu *et al.*, 2019), while the selection of mtDNA is due to the presence of mtDNA in hundreds to thousands of copies per cell and it will be advantageous in analysis of very limited number of samples (Weedn *et al.*, 2018). The DNA templates were successfully isolated from raw meats and Satay products using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit.

Analysis of DNA's purity and concentration was determined using absorbance ratios at wavelength 260 and 280 ($A_{260/280}$) using the instrument of Tecan's NanoQuant Plate. The ratio values at 260 and 280 ($A_{260/280}$) can be used as a purity index of DNA in which DNAs with $A_{260/280}$ of 1.8–2.0 are considered pure and it is suitable to be used as a DNA template during real-time PCR analysis. In addition, $A_{260/280}$ <1.8 indicates that the isolated DNAs also contain protein, phenols, and carbohydrates, while $A_{260/280}$ > 2.0 indicates the isolated DNA contains RNA (Orbayinah *et al.*, 2020). Table 2 shows the purity indexes and concentrations of DNA isolated from raw meat and commercial Satay products. The isolated DNAs had purity indexes approaching 1.8–1.99 indicating that the isolated DNAs are pure and suitable to be used as DNA templates for further analysis using real-time PCR analysis.

The optimization of annealing temperatures (Ta) is an important factor to be considered during analysis using real-time PCR, since Ta is associated with the attachment of primer in DNA targets. The gradient temperature was evaluated to find the optimal Ta of the designed primer. A range of Ta of 48ºC–58ºC was examined, and finally, Ta of 57.4ºC was selected as the optimal temperature due to its capability to

Table 1. Primer D-loop 38 targeting mitochondrial D-loop fragment of *C. familiaris*.

Sequences	Tm (°C)	GC (%)	Length base pair	Secondary structure (kcal/mole)		
				Hairpin	Self-dimer	Cross-dimer
Forward: CCATCAACCCTTGCTCGTAAT	55.2	47.6	21	0.11	−3.9	−5
Reverse: GCCCTGAAGTAAGAACCAGATG	55.4	50	22	−0.6	−3.55	

Table 2. The concentration and purity of DNA isolated from raw meat and commercial goat's satay sample.

Samples	DNA concentration (ng/µl)	Purity index (A_{260}/A_{280})
CM	309.8	1.84
Goat meat	273.86	1.85
Tree shrew meat	117.3	1.80
Pig meat	134.19	1.80
Bengal's rat meat	132.4	1.81
Cow meat	217.01	1.82
Mackerel meat	211.5	1.94
Chicken meat	698.8	1.92
Frog meat	339.9	1.99
Satay #1	83.56	2.00
Satay #2	138.91	2.05
Satay #3	75.62	1.92
Satay #4	69.61	1.97
Satay #5	59.23	1.88
Satay #6	83.42	2.04
Satay #7	97.08	1.94
Satay #8	25.72	1.96
Satay #9	86.64	1.94
Satay #10	117.25	1.92

provide the lowest values of Ct as shown in Figure 1A. From melting curve analysis (MCA), the suitability of 57.4ºC as annealing temperature was supported by the presence of a single peak in the amplification curve which means that there were no prime dimers and non-specific products observed during PCR amplification (Fig. 1B). Furthermore, this Ta was used for further analysis including validation of real-time PCR and the use of validated methods for analysis of commercial Satay products.

The validation method of real-time PCR method using SSP targeting on D-loop 38 was carried out by assessing some validation parameters which include primer specificity, efficiency, sensitivity, and repeatability. Primer specificity test was carried out using optimal Ta (57.4ºC) by amplifying the isolated DNAs from different animal tissues (canine, goat, cow, Bengal's rat, chicken, frog, mackerel, pork, and tree shrew) along with negative control or NTC. Figure 2A exhibited the amplification curve of real-time PCR using primer D-loop, in which only DNAs isolated from CM could be amplified with Ct value of 11.84 and relative fluorescence unit of 521. Figure 2B exhibited the MCA of the amplification curve in which only a single peak was observed above the threshold line belonging to CM. From the amplification curve and MCA, it can be concluded that SSP targeting on D-loop was specific for DNAs isolated from CM and the primer did not amplify other DNAs used. Efficiency and sensitivity tests were carried out by preparing a 2-fold serial dilution of pure canine DNA and followed by making a standard curve correlating between log concentration of DNA (*x*-axis) and Ct-values (*y*-axis) to find the efficiency (*E*-value).

The amplification profile of DNAs isolated from CM with 8 different concentrations is shown in Figure 3A. A good linear relationship existed with R^2 of 0.999, the slope of −3.110, *y*-intercept of 28.515, with efficiency value (*E*) 109.7% (Fig. 3B). CAC (CAS, 2010) declared the acceptance criteria for validation parameters of quantitative PCR assay is $R^2 \geq 0.98$ and *E* value of 90%–110% (Rohman *et al.*, 2020). Therefore, the *E*-value and R^2 met the requirements. LOD value was analyzed from eight different concentrations of DNA (25,000; 12,500; 6,250; 3,125; 1,562.5; 781.25; 390.63; 195.32 pg/µl), and then the amplification curve was obtained. The smallest concentration (195.32 pg/µl) can be still amplified by Real-time PCR above the threshold limit, therefore, DNAs with concentrations of 1,000, 500, 250, 125, 62.5, 31.25, 15.6, and 7.8 pg/µl were used. From the amplification curve, the LOD value obtained is 31.25 pg/µl, as shown in Figure 4.

The precision of real time PCR was evaluated by repeatability (intra-assay) test. The DNAs isolated from

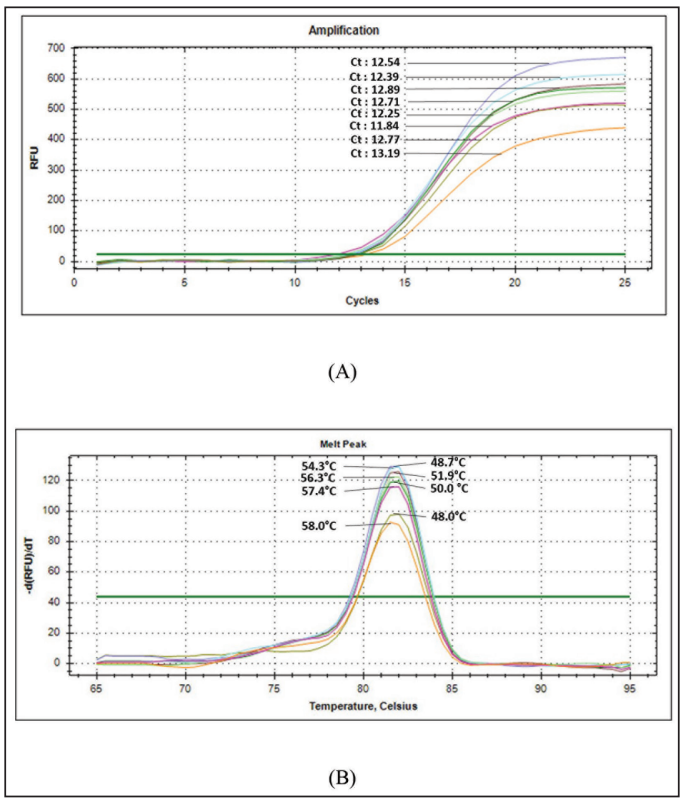

Fig. 1. Amplification curve during the optimization of annealing temperature at different temperatures. (A) = Amplification curve; (B) = melt peak curve.

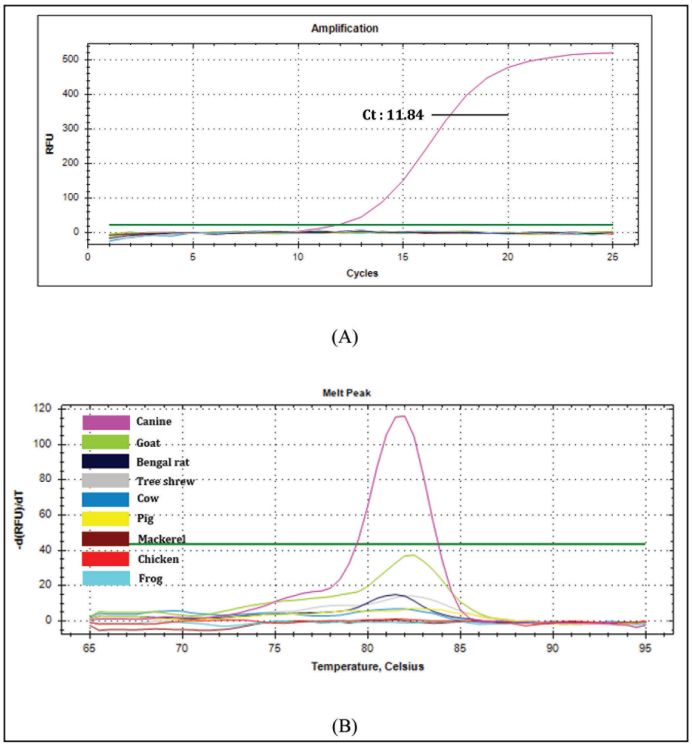

Fig. 2. The specificity test of primer D-loop 38 using DNAs of CM and 8 other meat species. (A) = Amplification curves; (B) = Melting peak curve.

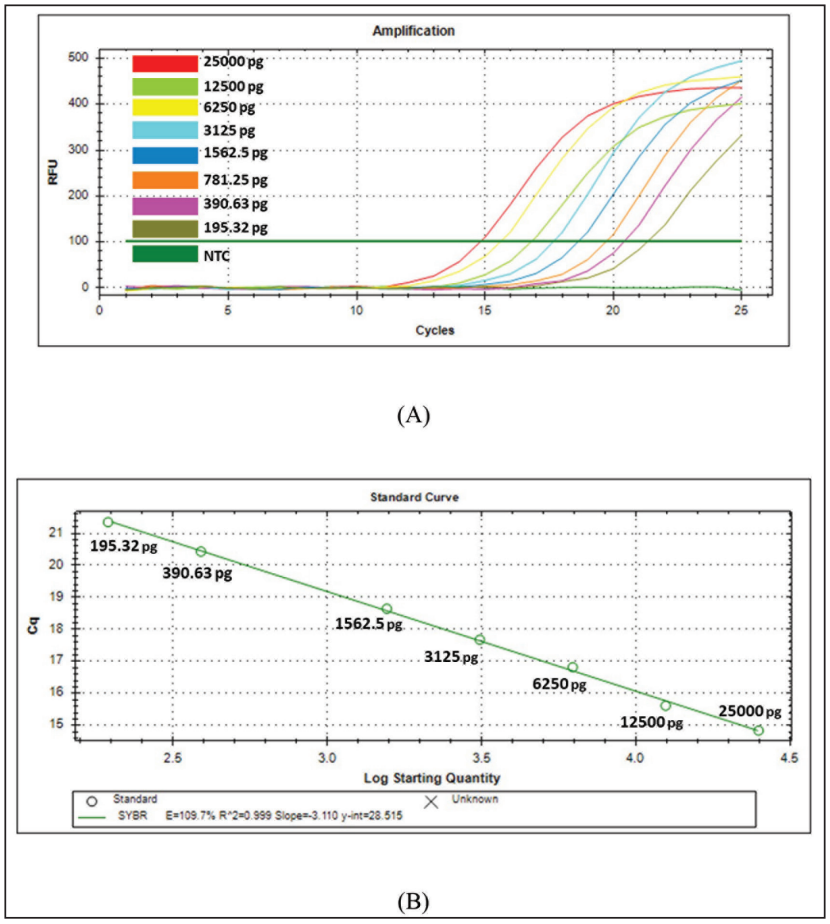

Fig. 3. Real-time PCR amplification using primer D-loop 38 at different DNA concentrations of CM with serial dilutions. (A) = Amplification curves; (B) = Standard curve correlating between log DNA concentration (x = axis) and Ct (y-axis).

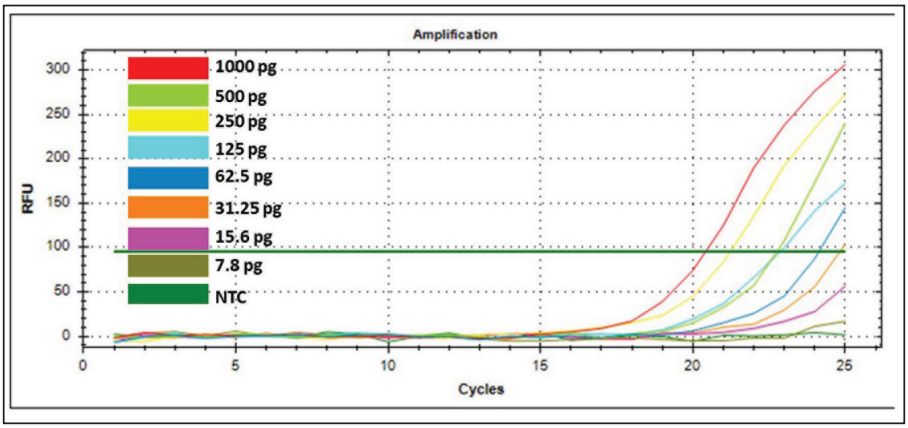

Fig. 4. Amplification of Canine's DNA at different DNA concentrations with serial dilution for sensitivity test using primer D-loop 38 of *C. familiaris*.

CM at a concentration of 6,250 pg/µl were amplified using SSP D-loop primer 38, and the Ct values were evaluated from eight replicates. The relative standard deviation (RSD) was used for the evaluation of repeatability, and RSD values of Ct were found to be 2% which is lower than the maximum RSD values set by CAC, i.e., ≤25%, therefore the RSD values met the acceptance criteria for precision parameter. The

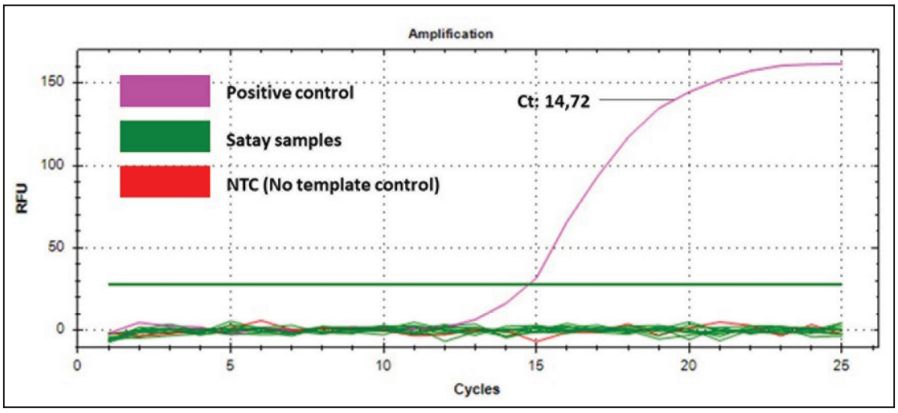

Fig. 5. The amplification curve from real-time PCR methods using primer D-loop 38 for analysis of 10 commercial goat's satay samples.

validated real-time PCR method using primer D-loop 38 was then applied for the detection of CM in 10 commercial goat's Satay around Yogyakarta, Indonesia, as shown in Figure 5. There were 10 samples along with positive control (CM's DNA) and NTC evaluated, and this validated method shows that only the DNA template of CM (positive control) was amplified with Ct value of 14.72, while all the commercial goat's satay and NTC (negative control) shows no amplification response. It can be concluded that all the evaluated commercial goat's satay samples did not contain CM.

The Realtime PCR methods using primer D-loop 38 have been successfully developed and validated to identify and confirm the presence of CM in commercial goat's satay. The design used in this method allows it to be further developed to detect other non-halal meat such as rats, pigs, monkeys, and so on. In addition, this method can also be developed in combination with other methods or approaches such as metabolomic approaches to improve the ability to detect products which has more complex characteristics like pharmaceutical products and cosmetic products.

Conclusion

The SSP targeting mitochondrial D-Loop 38 could specifically amplify DNAs isolated from CM using the optimized annealing temperature of 57.4ºC. Real-time using this primer was valid as indicated by accepted validation criteria. This method is sensitive enabling the detection of canine's DNAs as low as 31.25 pg/µl DNA. This validated method shows canine's DNA was not found in all commercial goat's satay samples. The development RT-PCR methods using primer D-loop 38 can be used for the identification and confirmation of CM in food products to support the development of a standard method for halal authentication analysis.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Authors' contributions

LAL: Conceptualized the research, designed the experiments, reviewing the manuscript; WNA: Collected the data, performed the experiments, conducted statistical analysis, wrote the manuscript and participated in manuscript revisions. MDL: Conceptualized the research, designed the experiments, wrote the manuscript and participated in manuscript revisions; YE: Conceptualized the research, designed the experiments, wrote the manuscript and participated in manuscript revisions; AAMBH: Designed the experiments, wrote the manuscript and participated in manuscript revisions; MZA: Designed the experiments, wrote the manuscript and participated in manuscript revisions; NH: Designed the experiments, wrote the manuscript and participated in manuscript revisions; MJB: edited the paper and participated in manuscript revisions. AR: Conceptualized the research, designed experiments, supervised students, edited the paper and participated in manuscript revisions.

Data availability

All data supporting the findings of this study are available within the manuscript.

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