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Application of Real-time PCR with Mitochondrial D-Loop Specific Primers for Identifying Bovine Adulteration in Meatball Products

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Abstract: The research aimed to develop an analytical approach utilizing Real-time PCR and a specific primer for the examination of bovine DNA in meatballs, addressing the issue of non-halal meat being used in halal meatballs. Real-time PCR enables rapid, specific, and sensitive detection, allowing for qualitative and quantitative species identification in processed products. The research involved designing specific primers for bovine DNA using IDT software, followed by DNA isolation and testing for various parameters such as specifications, linearity, detection limit, efficiency, and repeatability. The findings indicated that the primer D-Loop 922 (forward: 5-ATTACCATGCCGCGTGAA-3', Reverse: 5'-GATGAGATGGCCCTGAAGA AA-3'), designed and tested in silico using Primer-BLAST software from NCBI, effectively identified bovine DNA in both fresh meat and meatballs at an optimum annealing temperature of 59.5°C. The real-time PCR method utilizing the D-loop 922 primer successfully amplified bovine DNA in both bovine samples and bovine meatballs at a minimum concentration of 1 ng, with coefficients of variation (CV) of 0.20% for bovine DNA and 0.22% for bovine meatballs. Consequently, the D-loop 922 primer met the testing criteria and can be utilized to authenticate the halal status of meatball products, supporting the implementation of Law No. 33 of 2014 regarding halal certification.

Keywords: Bovine, Real-time PCR, D-Loop mtDNA, Meatball, Adulteration

1. INTRODUCTION

The topic of meat being a widespread necessity in society has led to an increased demand, resulting in a concerning issue of meat adulteration. With meat and meat-based foods, such as meatballs and sausages, being recognized as valuable sources of essential nutrients like proteins and vital fatty acids necessary for human growth [1], the demand for these products has surged not only in developed nations but also in emerging countries like Indonesia and Malaysia [2].

Meatballs are a favorite meat-based meal among many Indonesians. Meat is regarded as a key ingredient in food products since it can come from non-halal animals or halal animals that have not been slaughtered in accordance with Syariah rules [3]. Non-halal meat, including as pork, rat, and dog meat, is becoming increasingly popular in Indonesia, and multiple examples of unethical

merchants substituting or mixing halal meatballs with non-halal meat have been documented [4]. To validate the halal meatballs in circulation, an investigation of bovine DNA in meatballs is required. The availability of a real-time PCR technology that allows for quick, specific, and quantitative identification of a species in processed goods enables qualitative and quantitative species identification in processed products. Qualitative and quantitative identification of a species in processed products is possible with the existence of a real-time PCR method that allows rapid, specific, and sensitive detection. One of the nations with the greatest Muslim populations in the world is Indonesia, so Indonesia has required that food products distributed and sold in Indonesia must be halal certified [5]. The use of validated and standardized methods must be applied to ascertain whether the food product originates from bovine.

Various analytical methods have been suggested and applied for the analysis of meat, encompassing the examination of distinct markers, fingerprint profiles, and metabolic strategies. Gas chromatography (GC), including two-dimensional GC coupled with mass spectrometry, has proven effective in detecting meat through the identification of specific fatty acid markers [6]. However, this method requires sophisticated instrumentation and substantial investments, making it unsuitable for routine analysis in authenticating halal food products. Fingerprint profiles based on spectroscopic techniques, such as FTIR spectroscopy [7], Raman spectroscopy [8], and NMR spectroscopy [9], have also been reported. Nonetheless, the analysis of data derived from these molecular spectra often necessitates complex data processing. In recent years, the use of a metabolic approach based on high-resolution liquid chromatography-mass spectrometry has shown promise in identifying halal and non-halal meat. However, the obtained metabolites tend to be highly complex, making data interpretation challenging [10]. Considering these factors, the DNA-based method stands out as the preferred choice for meat analysis due to its specificity and sensitivity [11].

Real-time polymerase chain reaction (RT-PCR) is acknowledged as a gold standard for the detection of DNA in food products derived from meat, demonstrating both acceptable specificity and sensitivity. This is attributed to the fact that each meat species possesses unique DNA. RT-PCR provides efficient and effective direct quantitative analysis of DNA in food products [12]. DNA structure is highly stable at high temperature, therefore, DNA analysis is suitable in food products subjected to high thermal processing, while proteins can be denatured during the processing, rendering them unsuitable analytes for reliable identification and DNA is present in the cells of virtually every species [13]. Identification of meat to ensure halal status with real-time PCR offers several advantages, including specificity and reproducibility, making this method popular for diagnostic purposes [14].

Analysis of beef on capsule shells was once performed using the primer rRNA-12S mtDNA (Forward: CCC AAG CTA ACA GGA GTA CG, Reverse: TAG TGC GTC GGC TAT TGT AG) with good sensitivity and amplification efficiency [15]. Previous studies have also carried out the detection of beef by multiplex PCR using specific primers targeted to Cyt b mtDNA [16]. In this research, a RT-PCR method will be developed using a new specific primer on the D-Loop mtDNA to identify beef in meatballs, which has never been done in previous studies. The research was started by designing specific primers using IDT software. Then, the primers will be tested for specificity, linearity, limit of detection, efficiency, and repeatability. After the method is validated, the primer can be used for beef analysis and protects consumers from counterfeiting.

2. MATERIALS AND METHODS

2.1. Primer Designing

A set of primers, specifically targeting the D-loop fragment of bovine mitochondrial DNA, was created through the use of software from IDT (Integrated DNA Technologies, California, USA). NCBI (http://www.ncbi.nlm.nih.gov) provided the DNA sequences for the D-Loop of *Bos taurus* mitochondrial DNA. A set of primers was chosen from the five candidate primers acquired from IDT after completing an in-silico specificity test utilizing BLASTn (Basic Local Alignment Search Tool Nucleotide) features from the NCBI website.

2.2. DNA Isolation

DNA from raw meats was isolated using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen, Taiwan). The steps of the technique, which included cell lysis, protein degradation, DNA binding with silica matrix, contaminant washing, and DNA elution from silica matrix were all performed in line with the manufacturer's instructions. To measure DNA concentration and purity, the eluate or DNA isolate was examined qualitatively and quantitatively at 260 and 280 nm wavelengths using NanoQuant Spark Tecan (Switzerland). Pure DNA is defined as having an A260/A280 ratio between 1.8 and 2.0.

2.3. Analysis using Real-time PCR

The amplification of the specified DNA was executed using a PCR CFX96 system (Biorad, United States) with a concluding volume of 10 μL containing 5 μL of 2x SensiFast SYBR No-Rox Kit (Meridian Bioscrience, USA), 0.4 μL forward primer and 0.4 μL forward primer (Concentration at the end is 0.4 μM), 1 μL DNA with concentration of 10 ng/ μL, and 3,2 μL nuclease free water. In the temperature protocol, the process commenced with an initial pre-denaturation step at 95°C lasting 3 minutes, succeeded by 25 cycles of denaturation at 95°C lasting 5 seconds each. The optimal temperature range for annealing was 51.2 to 61.2°C, with 10 seconds at each temperature. The elongation step was established at 72°C for a duration of 20 seconds.

2.4. Validation of Real-time PCR-SSP

The performance of validation factors such as specificity, linearity, efficiency value, detection limit (LoD), and repeatability were used to validate the proposed primer. Each parameter's acceptance criteria were based on Codex Alimentarius. The validated approach was subsequently utilized to examine meatballs that are sold commercially items in Yogyakarta, Indonesia.

RESULTS AND DISCUSSION

3.1. Primer Designing

Analysis of bovine in food products can be performed using a DNA-based method using Real-time PCR because it is a specific and sensitive method. The purpose of this research is to develop an analytical method with Real-time PCR with specific primers for the analysis of bovine in meatballs. Primers are designed to target the mitochondrial D-Loop fragment of *Bos taurus* because of its high evolutionary level and prevalence of mitochondrial DNA, making it suitable for searching for different species and can increase specificity [17], [18]. Based on the database, a pair of primers was selected from 5 pairs of primers generated by considering the proportion of GC, 3′ end self dimer, melting temperature (Tm) and amplicon length. Selected primer specifications can be seen in Table 1.

Table 1. Primer D-loop 443 targeting D-loop fragment on mitochondrial DNA of *Bos Taurus*

Primer sequence 5′ – 3′	Tm (°C)	GC content (%)	Amplicon length
ATTACCATGCCGCGTGAA	62	50	133 bp
GATGAGATGGCCCTGAAGAAA	62	47,6	

3.2. DNA isolation

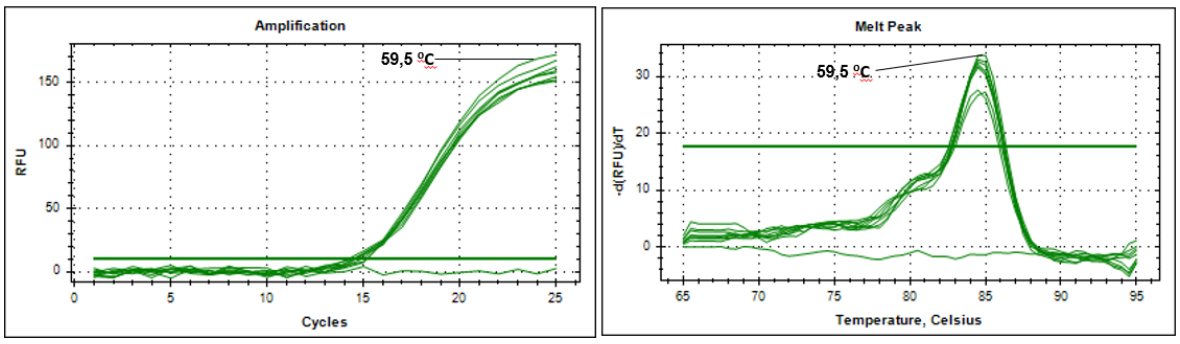
DNA isolation was carried out on fresh meat and bovine meatballs. DNA quantification was measured using the Nanoquant Spark Tecan with an absorbance of 260 and 280 nm (A260/A280). The wavelength ratio range of 260/280 nm indicating good purity is 1.8 to 2.0. A ratio value of less than 1.8 indicates the existence of protein impurities within the DNA isolates, while a ratio value of 260/280 nm more than 2.0 indicates the presence of RNA contaminants. Table 2 shows the purity and concentration of DNA.

Table 2. Concentration and purity evaluation of isolated DNA from raw meat and bovine meatball.

Samples	DNA concentration (ng/µL)	Purity index (A260/A280)
Canine	182.47	1.96
Chicken	85.44	1.93
Pork	137.66	1.92
Wild Boar	186.97	2.07
Goat	154.57	1.92
Bovine	60.57	1.96
Rat	75.49	1.94
Bovine Meatball	141.48	2.05

3.3. Annealing temperature optimization

Optimization of the primer annealing temperature (Ta) that has been designed for bovine DNA was carried out in the annealing temperature range using Real-time PCR. The annealing temperature range was obtained from the primary design data (Ta = Tm - 5°C). According to Rohman et al. (2020), generally the primary attachment temperature in a PCR reaction is 3-5°C lower than the melting temperature (Tm) of the primer pair [14]. Ta was optimized between 51.2 and 61.2°C. The 59.5°C annealing temperature was chosen because it produces the highest intensity with the lowest quantification cycle (Cq) value (Figure 1). This temperature will be used in the following validation procedure.



(a) **(b)**

Figure 1. Amplification curve of annealing temperature optimization at different temperatures and optimum at 59.5°C: (a) amplification curves; (b) melting peak

3.4. Spesificity

A specificity test was conducted to assess the selectivity of the designed primer towards the DNA of the target species. The assay utilized DNA samples from various species, including canine (*Canis familiaris*), chicken (*Gallus gallus*), pork (*Sus scrofa domesticus*), wild boar (*Sus scrofa*), goat (*Capra hircus*), bovine (*Bos taurus*), and rat (*Rattus novergicus*). The results of the specificity tests revealed that the D-Loop 922 primer specifically amplified bovine DNA, while showing no amplification with the DNA samples from the other six species (Figure 2). In bovine samples, the D-Loop 922 primer exhibited a relative fluorescence unit (RFU) value of 85.82 at an annealing temperature of 59.5°C, with CT values of 14.24 and a melting temperature (Tm) of 84.50°C.

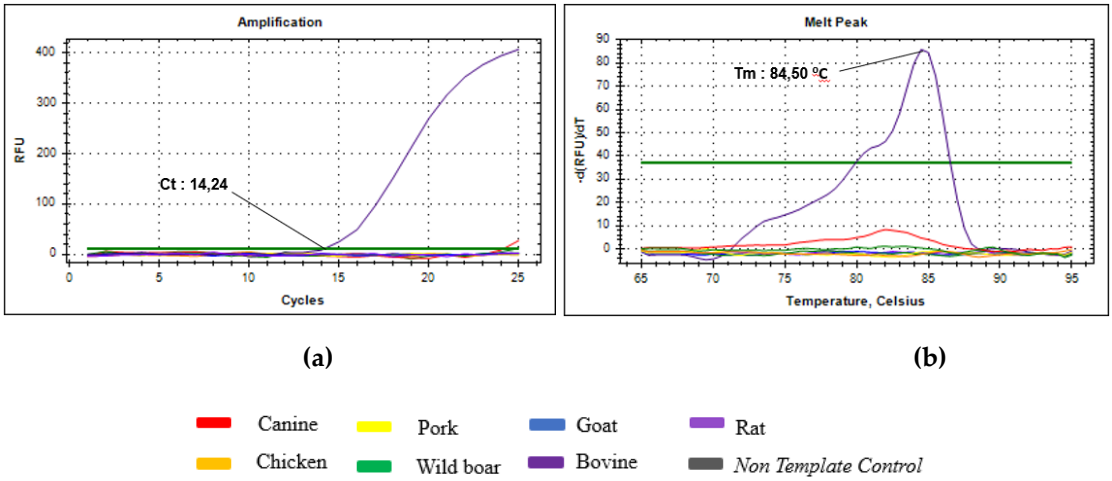


Figure 2. The specifity test of primer D-Loop 922 targeting D-Loop fragment of mitochondrial DNA of bovine tested with 6 other species sequences: (a) Amplification curves; (b) Melting peak

3.5. Sensitivity

The assays sensitivity was evaluated by conducting a tenfold dilution series of bovine DNA and bovine meatball DNA, ranging from 10-0.0001 ng, to establish the absolute detection limit (LoD). The LoD was was established by assessing the existence of amplification curves. The results indicated that the LoD for detecting bovine DNA and bovine meatball was 1 ng, which represented the lowest concentration capable of generating an amplification curve. No amplification curves were observed when DNA concentrations fell below 1 ng (Figure 3).

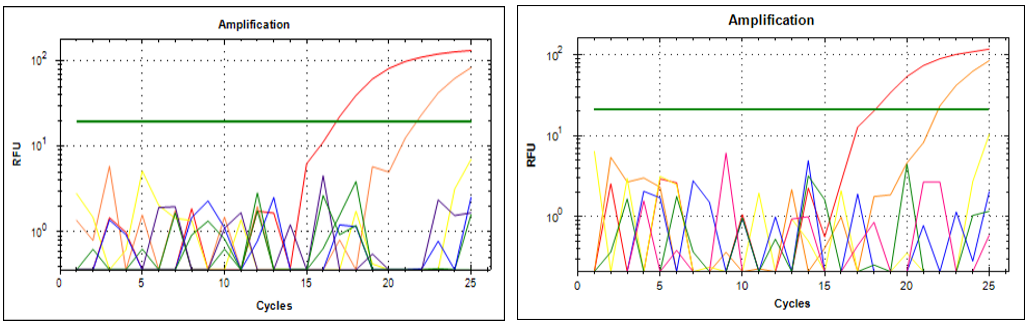


Figure 3. Amplification at different DNA concentration for sensitivity test using D-loop 922 primer of Bos Taurus: (a) Bovine; (b) Bovine meatball

The PCR methods' effectiveness was determined by executing a 2-fold serial dilution of pure bovine DNA and bovine meatball DNA. To calculate the efficiency (E) value, the amount of DNA was plotted against the Cq value of each concentration to create a standard curve. A good linear regression was produced from the standard curve of the amplification reaction to the DNA isolate of bovine and bovine meatball (Figure 4), with a high correlation coefficient (R) value 0.995, slope -3.882, y-intercept 34.256, and efficiency value (E) 81.0%. In the case of bovine meatballs, the R value is 0.994, the slope is -3.862, the y-intercept is 32,903, and the efficiency value (E) is 81.5%. R value above the acceptable requirement (R 0.98), so that the curve satisfies the sufficiently good linearity criteria. However, the efficiency value for D-Loop 922 primer is less than the recommended criteria (R $\geqslant$ 0.98, and E 90-110%) [19].

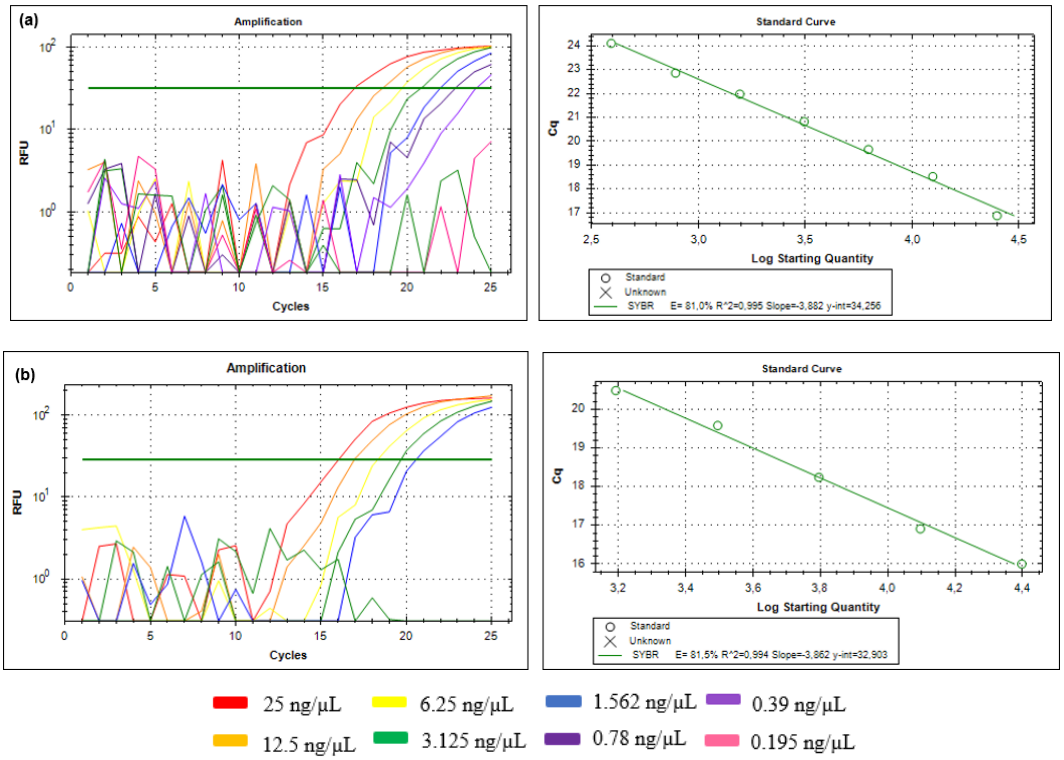
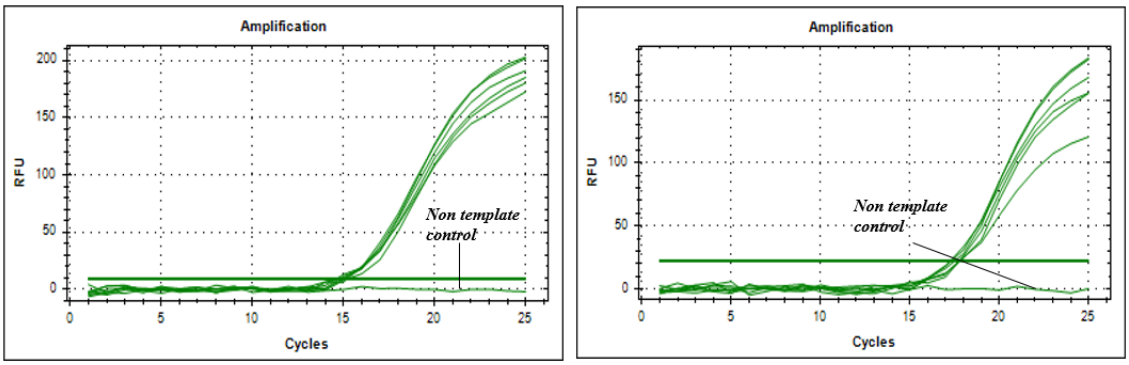


Figure 4. Amplification curves and standard curves of D-loop 922 primer with serial dilution of DNA template for efficiency analysis : (a) bovine; (b) bovine meatball

3.6. Repeatability

The variations in the PCR amplicon Tm in our assay are shown in figure 5. The qPCR assay was performed with 10 ng of bovine DNA and bovine meatball DNA. Cq value of bovine DNA is 15.05, 14.64, 15.23, 14.94, 14.83, and 15.29. Whereas Cq value ofbovine meatball DNA is 17.75, 17.20, 17.70, 17.25, 17.45, 17.71. The repeatability of bovine DNA with a coefficient of variation (CV) were 0.20%, whereas bovine meatball DNA is 0.22%. This result demonstrating a good reproducibility of the developed method. CV values of bovine DNA and bovine meatball DNA have met the criteria recommended in the use of method PCR (CV≤25%) [19], [20].



(a) **(b)**

Figure 5. Amplification using D-loop 922 for repeatability analysis. (a) bovine; (b) bovine meatball

3. CONCLUSION

The D-loop 922 primers exhibit the capability to detect the presence of bovine DNA. This assay is highly versatile and can be applied for the authentication of processed adulterated meat products. Furthermore, when used at an optimal annealing temperature of 59.5°C, this system demonstrates the ability to amplify minimal amounts of DNA, with an absolute limit of detection of 1 ng DNA. The assay also demonstrates acceptable correlation values (R of 0.995 and 0.994) and precision (RSD of 0.20% and 0.22%). This method can be used to identify the presence of beef and is promising for adoption in advanced laboratories in Indonesia.

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Conflicts of interest: The authors declare no conflict of interest.

References

[1] Y. Kumar and S. Chandrakant Karne, "Spectral analysis: A rapid tool for species detection in meat products," *Trends in Food Science & Technology*, vol. 62, pp. 59–67, Apr. 2017, doi: 10.1016/j.tifs.2017.02.008.

[2] Y. Erwanto, M. Z. Abidin, E. Y. P. M. Sugiyono, and A. Rohman, "Identification of Pork Contamination in Meatballs of Indonesia Local Market Using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) Analysis," *Asian-Australas J Anim Sci*, vol. 27, no. 10, pp. 1487–1492, Oct. 2014, doi: 10.5713/ajas.2014.14014.

[3] S. Sultana, M. A. M. Hossain, A. Azlan, M. R. Johan, Z. Z. Chowdhury, and Md. E. Ali, "TaqMan probe based multiplex quantitative PCR assay for determination of bovine, porcine and fish DNA in gelatin admixture, food products and dietary supplements," *Food Chemistry*, vol. 325, p. 126756, Sep. 2020, doi: 10.1016/j.foodchem.2020.126756.

[4] M. E. Ali *et al.*, "Multiplex PCR assay for the detection of five meat species forbidden in Islamic foods," *Food Chem*, vol. 177, pp. 214–224, Jun. 2015, doi: 10.1016/j.foodchem.2014.12.098.

[5] Presiden RI, "UU No. 33 Tahun 2014." 2014. Accessed: Dec. 08, 2023. [Online]. Available: http://peraturan.bpk.go.id/Details/38709/uu-no-33-tahun-2014

[6] D. Indrasti, Y. B. Che Man, S. Mustafa, and D. M. Hashim, "Lard detection based on fatty acids profile using comprehensive gas chromatography hyphenated with time-of-flight mass spectrometry," *Food Chemistry*, vol. 122, no. 4, pp. 1273–1277, Oct. 2010, doi: 10.1016/j.foodchem.2010.03.082.

[7] W. S. Rahayu, A. Rohman, S. Martono, and S. Sudjadi, "Application of FTIR Spectroscopy and Chemometrics for Halal Authentication of Beef Meatball Adulterated with Dog Meat," *Indonesian Journal of Chemistry*, vol. 18, no. 2, Art. no. 2, May 2018, doi: 10.22146/ijc.27159.

[8] M. Mortas, N. Awad, and H. Ayvaz, "Adulteration detection technologies used for halal/kosher food products: an overview," *Discov Food*, vol. 2, no. 1, p. 15, Apr. 2022, doi: 10.1007/s44187-022-00015-7.

[9] A. Pajuelo *et al.*, "1H NMR to analyse the lipid profile in the glyceride fraction of different categories of Iberian dry-cured hams," *Food Chemistry*, vol. 383, p. 132371, Jul. 2022, doi: 10.1016/j.foodchem.2022.132371.

[10] Suratno, A. Windarsih, H. D. Warmiko, Y. Khasanah, A. W. Indrianingsih, and A. Rohman, "Metabolomics and Proteomics Approach Using LC-Orbitrap HRMS for the Detection of Pork in Tuna Meat for Halal Authentication," *Food Anal. Methods*, vol. 16, no. 5, pp. 867–877, May 2023, doi: 10.1007/s12161-023-02472-x.

[11] Y.-H. P. Hsieh and J. A. Ofori, "Speciation of animal fat: Needs and challenges," *Crit Rev Food Sci Nutr*, vol. 57, no. 8, pp. 1673–1680, May 2017, doi: 10.1080/10408398.2015.1012291.

[12] K. Dolch, S. Andrée, and F. Schwägele, "Comparison of Real-Time PCR Quantification Methods in the Identification of Poultry Species in Meat Products," *Foods*, vol. 9, no. 8, Art. no. 8, Aug. 2020, doi: 10.3390/foods9081049.

[13] N. Z. Ballin, "Authentication of meat and meat products," *Meat Science*, vol. 86, no. 3, pp. 577–587, Nov. 2010, doi: 10.1016/j.meatsci.2010.06.001.

[14] A. Rohman, S. Orbayinah, A. Hermawan, S. Sudjadi, A. Windarsih, and S. Handayani, "The development of real-time polymerase chain reaction for identification of beef meatball," *Applied Food Research*, vol. 2, no. 2, p. 100148, Dec. 2022, doi: 10.1016/j.afres.2022.100148.

[15] N. E. Fitriani, "Analisis DNA Sapi dengan Primer rRNA-12S Mitokondria dan Aplikasinya dalam Deteksi DNA Sapi pada Cangkang Kapsul Gelatin Menggunakan Real-time PCR (Polymerase Chain Reaction)," Universitas Gadjah Mada, 2016. Accessed: Dec. 08, 2023. [Online]. Available: https://etd.repository.ugm.ac.id/penelitian/detail/101128

[16] R. P. Rudi, H. suryandani Hendy, D. ahmad hudaya Dadan, E. yuniarsih Eko, and T. R. Tuti, "Implementasi Metode Multiplex untuk Deteksi Daging Sapi: Implementasi Metode Multiplex untuk Deteksi Daging Sapi," *JURNAL PERTANIAN & INDUSTRI PANGAN*, vol. 2, no. 1, Art. no. 1, Aug. 2022.

[17] K. Chen, T. Baxter, W. M. Muir, M. A. Groenen, and L. B. Schook, "Genetic Resources, Genome Mapping and Evolutionary Genomics of the Pig (Sus scrofa)," *Int J Biol Sci*, vol. 3, no. 3, pp. 153–165, Feb. 2007.

[18] N. Karabasanavar, P. S. Girish, D. Kumar, and S. P. Singh, "Detection of beef adulteration by mitochondrial D-loop based species-specific polymerase chain reaction," *International Journal of Food Properties*, vol. 20, no. sup2, pp. 2264–2271, Dec. 2017, doi: 10.1080/10942912.2017.1369103.

[19] Codex Alimentarius Comissions, "CAC/GL 74-2010 Guidelines on performance criteria and validation of methods for detection, identific-CAC Standards-International-Food Laws & Regulations-Documents-Global FoodMate." 2010. Accessed: Dec. 08, 2023. [Online]. Available: http://files.foodmate.com/2013/files_1810.html

[20] P. Hübner, H. U. Waiblinger, K. Pietsch, and P. Brodmann, "Validation of PCR methods for quantitation of genetically modified plants in food," *J AOAC Int*, vol. 84, no. 6, pp. 1855–1864, 2001.