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Design and Performance Test of Specific Primers to Detect Bovine DNA Fragments using Multiplex PCR Technique for Halal Authentification

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Abstract: *Adulterating meat products with several species, including non-halal species, is often found in commercial products. This study aims to design and validate the Cytochrome c oxidase subunit I (CO1) primers to detect the non-halal species. A pair of species-specific primers encoding the CO1 gene were designed to amplify bovine DNA, tested for specificity, and applied in multiplex polymerase chain reaction (PCR) technique with D-loop primers for pigs, Cyt-b for rats, and 12S rRNA for dogs. The CO1 primers, along with D-loop primers for porcine, Cyt-b primers for rats, and 12S rRNA primers for dogs can be used to detect specific bovine DNA with a size of 279 bp and sequence similarity of 96%. In addition, dog, rat, and porcine DNA were amplified at 101, 603, and 951 bp, respectively. These four primers are specific and can amplify the target DNA to detect non-halal meat component contamination in a single reaction process.*
Keywords: *bovine, CO1 primer, halal authentification, meat products, multiplex PCR*

1. Introduction

Food adulteration is often found in food processing (Esteki et al., 2019; Huq et al., 2022; Tibola et al., 2018). Foodstuff is adulterated for various reasons, including increasing margin, carelessness, or covering up defects that might occur in the food (Banti, 2020; Choudhary et al., 2020; Sharma et al., 2017). The meat mixed into the claimed food product is usually of poor quality and unfit for consumption or forbidden by certain religions, such as pork, rat, dog, or other non-halal meat (Manalu et al., 2019; Salahudin et al., 2018). Adulteration through the addition of non-halal ingredients is a major concern for Muslims. Every Muslim is forbidden from using non-halal ingredients or eating non-halal food (Denyingyhot et al., 2022; Halimi et al., 2021). Meanwhile, it is not easy to distinguish non-halal mixed meat from beef, so developing a reliable detection technique for mixing ingredients needs is necessary (Prachugsorn et al., 2022; Saputra et al., 2018).

Several detection techniques for food adulteration have been developed based on the presence of protein, fat, and DNA responses (Ali et al., 2012; Chappalwar et al., 2020; Yalçınkaya et al., 2017). DNA-based analysis is widely used because it is stable even under extreme conditions (not easily denatured) (Astill et al., 2019; Članjak – Kudra et al., 2021). It is suitable for detecting food products undergoing a series of production processes. The PCR technique using species-specific primers has been widely applied to detect adulteration of processed meat products because it can amplify specific target sequences (Kang & Tanaka, 2018; Vaithiyanathan et al., 2021). The PCR technique using species-specific primers has been widely applied to detect adulteration of processed meat products because it can amplify specific target sequences. Several species-specific mt-DNA primers that have been used to

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detect species origin are *ND2* (pork), *ATPase8* (horse), *ATPase6* (donkey) (Kesmen et al. (2007); as cited in Ai et al., 2019), *Cyt-b* (Matsunaga et al., 1999, as cited in Ni'mah et al., 2016), and *16S r*RNA (Cai et al., 2021), *ND1* (Raharjo et al., 2018). One of the developments of the PCR technique is the multiplex PCR technique which uses multiple primers and DNA samples in one reaction to amplify multiple target regions (Zhang et al., 2020). This technique detects multiple species in a single sample of each reaction. In multiplex PCR, each primer must be specific for a particular species, and the melting temperature (T_m) should be similar or nearly for the PCR to work properly (Kalendar et al., 2017).

Previously, Kusnadi et al. (2020) successfully tested the specificity of mt-DNA D-loop primers to detect porcine in various meat (pork, beef, goat, chicken, and lamb) using conventional PCR techniques. Several mt-DNA primers have been developed, including D-loop for porcine (Kusnadi et al., 2020), *Cyt-b* for rats (Nuraini et al., 2012), and *12S r*RNA for dogs (Chen et al., 2019). The CO1 primers were designed and validated in this study using the multiplex PCR technique, allowing forgery detection to be done quickly and accurately in a single test.

2. Materials and Methods

2.1. Bovine Specific Primer Design

A pair of bovine-specific primers targeting the CO1 gene in mt-DNA bovine were designed based on the sequence of CO1 gene obtained from the website of the National Center for Biotechnology Information (NCBI). The *Bos taurus* species with GenBank accession number MT576844.1 was selected as the nucleotide sequence reference for bovine. The bovine CO1 sequences were aligned with those of dog (*Canis lupus familiaris*, KJ522809.1), rat (*Rattus norvegicus*, NC 001665.2), and porcine (*Sus scrofa domesticus*, KJ789952.1) to obtain a specific and unique sequence. The selection of primer sequences is carried out by considering the size of the product amplification (100–500 bp), primer length 20–30 bp, GC 40–60%, melting temperature (T_m) 50–60°C, and annealing temperature (T_a) max 5°C from T_m.

2.2. DNA Isolation

The chloroform isoamyl-alcohol method was used to isolate DNA from meat samples (beef, porcine, rats, and dogs) with some modifications, including the elimination of phenol, storage time, and centrifugation speed (× g) (Sambrook et al., 1989, as cited in Priyanka et al., 2021). The samples of beef and pork were obtained from Malang city supermarkets. In contrast, the samples of dog and rat meat were supplied by The Central Laboratory of Life Sciences (LSIH) of Brawijaya University.

Each meat sample was weighed up to 20 mg and placed in a 1.5 mL Eppendorf tube containing 500 µL of STE buffer (NaCl, Tris-Cl pH 8, EDTA pH 8). First, the samples were mashed with a micro pestle and mixed with 40 µL of 10% SDS and 20 µL of proteinase-K 20 mg/mL. Next, they were vortexed for 20 seconds before being incubated in a thermal mixer at 55°C and 800 rpm (MHR 13, HLC BioTech). Finally, they were centrifuged for 10 minutes at 29°C at 16.000 × g (Mikro 22R, Hettich).

The amount of 400 µL of the supernatant was transferred to a new 1.5 mL Eppendorf tube and added with the same volume of chloroform and isoamyl alcohol with the 24:1 ratio. Then, 40 µL of 5M NaCl was added and mixed slowly. The mixture was centrifuged for 10 and 5 minutes at a temperature of 29°C and a speed of 16.000 × g. The procedure was repeated the second time without adding 5M NaCl. The supernatant was then transferred into a new 1.5 mL Eppendorf tube along with 800 µL of absolute cold ethanol and 40 µL of 5M NaCl. The samples were slowly mixed and incubated at -20°C for 2.5 hours before being centrifuged for 10 minutes at 4°C at 16.000 × g. Next, the pellet was added to 500 µL of 70% ethanol before being centrifuged for 5 minutes at 4°C at 16.000 x g. Finally, the pellets tube was placed in a Thermomix at 55°C until the ethanol evaporated. The pellet was then dissolved in 50 µL of pH 7.6 TE buffer. A NanoDrop™ spectrophotometer (NanoDrop/ND-1000 UV/Vis) was used to analyze the samples for purity and concentration.

2.3. PCR Amplification

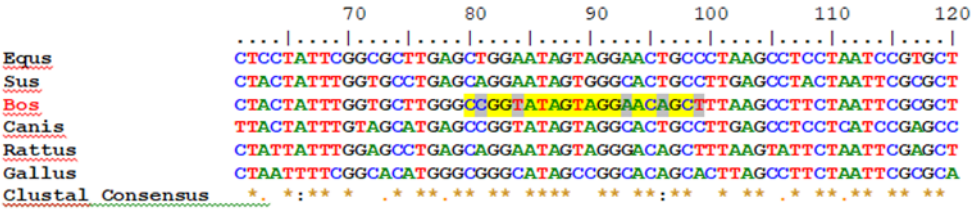
The multiplex PCR technique was used to test primer specificity. DNA isolates were amplified using *12S r*RNA primers for dogs, Cyt-b primers for rats, D-loop primers for porcine, and CO1 primers for bovine. In a 0.2 mL tube, the total volume of 20 µl PCR reaction mixture contained 10 µl My TaqTM HS Red Mix, 0.5 µl of forward and reverse primers (dogs, rat, porcine, and bovine), 3 µl of ddH_2O, 0.5 µl of DNA beef, 0.5 µl porcine DNA, 1 µl rat DNA, and 1 µl dog DNA. Spin down was used to homogenize this PCR mixture. Thermal cycler (CR system 9700, Applied Biosystems) set to 95°C for 5 minutes for hot start, then 35 denaturation cycles at 95°C for 30 seconds, annealing

at 55°C for all primers in 30 seconds obtained through PCR optimization of annealing and extension temperatures at 72°C for 40 seconds. The last stage is the final extension at 72°C for 5 minutes. The PCR products were stored at -20°C before being used for further analysis. PCR products were visualized by electrophoresis (Mupid 2 plus) technique using 1.5% agarose concentration dissolved in 1× TBE buffer and run at 50 V for 45-50 minutes. Electrophoresis results were observed using a ChemiDoc gel imaging device (BR-200, Bio-Rad). Amplification samples with bovine primers were sequenced at 1st Base Malaysia and analyzed using bioinformatics programs (sequencer 4.1.4) and Basic Local Alignment Search Tool (BLAST).

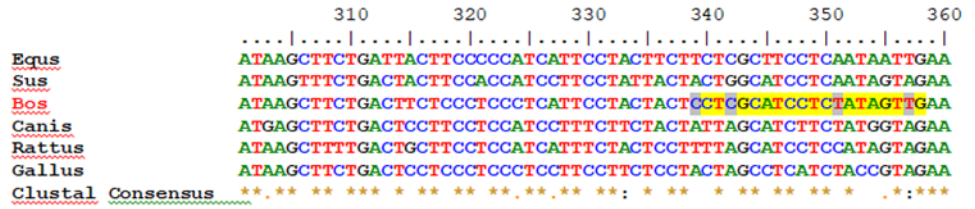
3. Results and Discussion

3.1. Bovine Specific Primer Design CO1

A pair of forward and reverse primers were designed manually based on the mt-DNA region of the bovine CO1 gene (*Bos taurus*) taken from the NCBI database with GenBank accession number MT576844.1. As a result, 1545 bp long bovine CO1 gene sequences were aligned with CO1 sequences from dogs, porcine, and rats, as shown in Figure 1.



(a)



(b)

Figure 1. Alignment of CO1 Gene Sequences for Bovine, Horse, Pig, Mouse, Dog, and Chicken Species. (a) Primer Forward, (b) Primer Reverse.

The higher phylogenetic group is related to the higher base variation in the nucleotide sequence (Hasibuan et al., 2017). The length of the primers was 20 bp, designed to amplify sequence targets of 279 bp length. The length of this target corresponds to the amplification product of other primer targets used in multiplex PCR. Shorter target DNA will be more stable and easier to obtain from the amplification of degraded template DNA, such as DNA in processed meat product samples. In general, processed meat products have gone through a series of processes that can damage DNA, such as refining raw materials and heating. The characteristics of the designed forward and reverse CO1 primers and several other primers used in this multiplex PCR technique as shown in Table 1.

Table 1. The Primer Pairs Used in Multiplex PCR Identification of Dog, Rat, Porcine, Bovine

Species	Sequence	Gene	Length (bp)	% GC	T_a (°C)	Amplicon (bp)	Reference
Dog	F: AATTGAATCGGGCCATGAA	*12S r*RNA	19	42	55	101	(Martín et al., 2007)
	R: CTCCTCTTTGTTTTAGTTAAGTTAA TCTG		30	30			
Rat	F: GACCTCCCAGCTCCATCAAACATC TCATCTTGA TGAAA	*Cyt-b*	38	44,7		603	(Matsunaga et al., 1999, as cited in Nuraini et al., 2012)
	R: GAATGGGATTTTGTCTGCGTTGGA GTTT		28	42,8			
Porcine	F: TACTTCAGGACCATCTCACC	D-loop	20	50		120	(Haunshi & Saxena, 2008, as cited in Kusnadi et al., 2020)
	R: TATTCAGATTGTGGGCGTAT		20	40			
Bovine	F: CCGGTATAGTAGGAACAGCT	CO1	20	50		279	This study
	R: CAACTATAGAGGATGCGAGG		20	50			

The CO1 mt-DNA primers designed qualify as as good primers. The 20 bp primer length is sufficient for binding the template DNA at the appropriate annealing temperature and obtaining specific sequences (Borah, 2011, as cited in Cahyadi et al., 2018). Therefore, a primer size that is too long will affect the annealing process, but a primer size that is too short will affect the primer's specificity. In addition, the CO1 primer has a guanine-cytosine (GC) content of 50%. Therefore, the percentage of primary GC should be between 40% and 60%. A high percentage of GC causes the formation of a hairpin structure, whereas the low one reduces the efficiency of the PCR (Sasmito et al., 2014).

3.2. DNA Isolation and Quantitative Measurement

It is important to ensure the purity and adequate concentration of DNA before carrying out the DNA amplification process using the PCR technique. Sufficient DNA concentration is needed for the DNA amplification process to occur properly (Piskata et al., 2017). Therefore, DNA isolates from meat samples were tested quantitatively, as shown in Table 2.

Table 2. The Results of DNA Isolation of Fresh Meat

Sample	Concentration (ng/µl)	Purity (λ 260/280)
Porcine	94.92	1.99
Bovine	138.11	1.92
Dog	41.88	2.06
Rat	143.37	2.01

The concentration of DNA templates required for the PCR process ranges from 10 to 100 ng/µl (Nugroho et al., 2017). It is the total concentration of DNA in the PCR mix. The DNA isolates obtained during the isolation process ranged from 41.88 ng/µl to 143.37 ng/µl. Therefore, the DNA concentration is adequate for use in the PCR reaction. The purity of dog and rat DNA is 2.06 and 2.01, respectively. It indicates little RNA contamination because the RNAse enzyme is not used in isolation (RNAse enzyme can degrade RNA).

3.3. Bovine Primer Specificity Test

3.3.1. Primer Specificity Test using single PCR

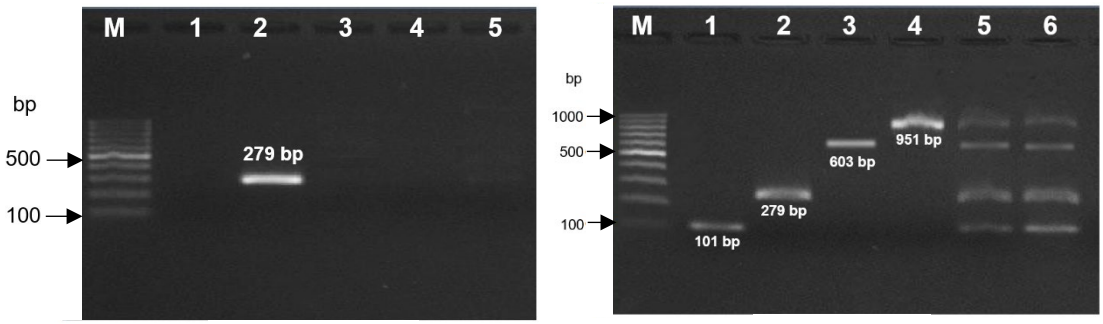
Primers' specificity is critical in food authentication studies to avoid misidentification. Therefore, the specificity of the primers was tested in this study by amplifying DNA extracted from beef (positive control), dog, porcine, rat, and ddH_2O (negative control). Figure 2a visualizes the CO1 single PCR primer specificity test.

The results showed that the DNA band formed was a single band with a size of 279bp, which corresponded to the target DNA (bovine), as shown in Figure 2a, line 2. The CO1 gene has several advantages over other genes in mt-DNA in high-level species identification and characterization studies. The CO1 gene's nucleotide sequence contains few deletions and insertions, indicating that many conserved regions can be used to identify species. Furthermore, the deletion or insertion of the nucleotide may result in point mutations, leading to silent mutations that do not result in amino acid changes. This nucleotide mutation may be sufficient to distinguish among species. As a result, the CO1 gene can be used as a genetic marker for precise species identification (Luo et al., 2011; Wirdateti et al., 2016). It was evidenced by the absence of DNA bands in non-target species and negative control.

3.3.2. Primer Specificity Test using Multiplex PCR

Multiplex PCR is a technique for detecting multiple DNAs in a single reaction. Therefore, specific primers are needed for each targeted DNA. Thus, it is critical to perform a primer specificity test to determine the specificity of the primer in amplifying each target DNA. Figure 2b visualizes the CO1 multiplex PCR primer specificity test.

Figure 2b shows that the primer can amplify the target DNA in each sample of singleplex PCR well. It is indicated by the formation of four bands corresponding to each size of the target species. DNA of dogs, bovine, rat, and porcine was successfully amplified at 101 bp, 279 bp, 603 bp, and 951 bp (line 1-4), respectively, based on the results of Martín et al. (2007), Nuraini et al. (2012), and Kusnadi et al. (2020). These results indicate that the four primers are specific primers capable of amplifying the target DNA. In addition, using the multiplex PCR technique, the mixed DNA (lanes 5 and 6) was successfully amplified in one reaction.



(a) (b)

Figure 2. (a).Visualization of Primer Specificity Test with Single PCR (M= DNA Ladder 100 bp, 1= ddH_2O, 2= Bovine, 3= Dog, 4= Porcine, 5= Rat). (b) Visualization of Primer Specificity Test with Multiplex PCR Type: M=DNA Ladder 100 bp (1= Dog, 2= Bovine, 3= Rat, 4= Porcine, 5 and 6= Mix DNA).

3.4. Bovine DNA Sequence Analysis

The amplification results with bovine-specific CO1 primers were analyzed using a bioinformatics program (sequencher 4.1.4) to see the target DNA sequences. Furthermore, the DNA sequences were analyzed using the BLAST program to find the homology of the primer sequence with DNA sequences published in the GenBank, as shown in Figure 3, resulting in similarity/identity values and query coverage. The DNA sequence amplified by CO1 primer has an amplicon length of 279 bp, matching the sequence located at 5764 - 6042 bp of 16339 bp of *Bos taurus* isolate YB6 mitochondrion with accession number MT576844.1. Based on the results of BLAST analysis, the primary sequence of CO1 gene amplification had 100% query cover, 96% identity, and a 3% total gap. The results demonstrated that the CO1 primer detected bovine DNA fragments with a high and specific match.

Score	Expect	Identities	Gaps	Strand
448 bits(242)	2e-121	269/280(96%)	9/280(3%)	Plus/Plus

```
Query  1     CCGGTATAGTAGGAACAGCTCTAAGCCTACTAAT-C-CGCTGAATTAGGCCAACCCGGAA  58
             ||||||||||||||||||||||||||||||||| ||||| | ||||||||||||||||||||||||
Sbjct  5764  CCGGTATAGTAGGAACAGCTCTAAGCCTTCTAATTCGCGCTGAATTAGGCCAACCCGGAA  5823

Query  59    CTCTGCTCGGAGACGACCAAATCTACAACGTAGTTGTAACCGCACACGCATTTGTAATAA  118
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5824  CTCTGCTCGGAGACGACCAAATCTACAACGTAGTTGTAACCGCACACGCATTTGTAATAA  5883

Query  119   TCTTCTTCATAGTAATACCAATCATAATTGGAGGATTCGGTAACTGACTTGTTCCCCTAA  178
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5884  TCTTCTTCATAGTAATACCAATCATAATTGGAGGATTCGGTAACTGACTTGTTCCCCTAA  5943

Query  179   TAATTGGTGCTCCCGATATAGCA-TTCCCCGAATAAATAAATATAAGCTTCTGACTCCAC  237
             ||||||||||||||||||||| |||||||||||||| |||||||||||||||||||| |
Sbjct  5944  TAATTGGTGCTCCCGATATAGCATTTCCCCGAATAAAT-AATATAAGCTTCTGACTCCTC  6002

Query  238   C-TCC-TC-TT--TACTACTCCTCGCATCCTCTATAGTTG  272
             | ||| || ||  ||||||||||||||||||||||||||||
Sbjct  6003  CCTCCCTCATTCCTACTACTCCTCGCATCCTCTATAGTTG  6042
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Figure 3. Results of DNA Sequence Query Compared with Sequences in GenBank.

4. Conclusion

Species-specific primers designed based on the bovine mt-DNA CO1 gene sequence were confirmed to detect bovine DNA with a size of 279 bp and sequence similarity of 96%. Therefore, CO1 primers can be applied for specific detection of bovine DNA along with D-loop primers for porcine, *Cyt-b* for rats, and *12S r*RNA for dogs. Therefore, these four primers can be applied to detecting counterfeiting and halal authentication.

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References

Ai, L., Liu, J., Jiang, Y., Guo, W., Wei, P., & Bai, L. (2019). Specific PCR Method for Detection of Species Origin in Biochemical Drugs Via Primers for The ATPase 8 gene by Electrophoresis. *Microchimica Acta*, *186*(9), 634–641. https://doi.org/10.1007/s00604-019-3738-5

Ali, M. E., Kashif, M., Uddin, K., Hashim, U., Mustafa, S., & Che Man, Y. Bin. (2012). Species Authentication Methods in Foods and Feeds: The Present, Past, and Future of Halal Forensics.

Food Analytical Methods, *5*(5), 935–955. https://doi.org/10.1007/s12161-011-9357-3

Astill, J., Dara, R. A., Campbell, M., Farber, J. M., Fraser, E. D. G., Sharif, S., & Yada, R. Y. (2019). Transparency in Food Supply Chains: A Review of Enabling Technology Solutions. *Trends in Food Science & Technology*, *91*, 240–247. https://doi.org/10.1016/j.tifs.2019.07.024

Banti, M. (2020). Food Adulteration and Some Methods of Detection, Review. *International Journal of Nutrition and Food Sciences*, *9*(3), 86–94. https://doi.org/10.11648/j.ijnfs.20200903.13

Cahyadi, M., Puruhita, Barido, F. H., & Hertanto, B. S. (2018). Specific Primer Design of Mitochondrial 12S rRNA for Species Identification in Raw Meats. *IOP Conference Series: Earth and Environmental Science*, *102*, 1–7. https://doi.org/10.1088/1755-1315/102/1/012038

Cai, Z., Zhou, S., Liu, Q., Ma, H., Yuan, X., Gao, J., & Cao, J. (2021). A Simple and Reliable Single Tube Septuple PCR Assay. *Foods*, *10*(5), 1–13. https://doi.org/10.3390/foods10051083

Chappalwar, A., Animal, M., Pathak, V., Goswami, M., & Sharma, B. (2020). Recent Novel Techniques Applied for Detection of Meat Adulteration and Fraudulent Practices. *Indian Journal Veterinary Public Health*, *7*(1), 1–6.

Chen, C., Lin, Y., Tao, R., Xia, R., Zhang, X., Zhang, J., Yang, Z., Li, C., & Zhang, S. (2019). An enhanced DNA Amplification Method to Detect the Species Origins of the Rootless Hair Shafts. *Forensic Science International: Genetics Supplement Series*, *7*(1), 647–648. https://doi.org/10.1016/j.fsigss.2019.10.124

Choudhary, A., Gupta, N., Hameed, F., & Choton, S. (2020). An overview of Food Adulteration: Concept, Sources, Impact, Challenges and Detection. *International Journal of Chemical Studies*, *8*(1), 2564–2573. https://doi.org/10.22271/chemi.2020.v8.i1am.8655

Članjak – Kudra, E., Fazlović, N., Alagić, D., Smajlović, M., Čaklovica, K., & Smajlović, A. (2021). An Overview of Most Commonly Used Methods for Detection of Fish Mislabeling. *Veterinaria*, *70*(2), 169–184. https://doi.org/10.51607/22331360.2021.70.2.169

Denyingyhot, A., Srinulgray, T., Mahamad, P., Ruangprach, A., Sa-I, S., Saerae, T., Vesaratchavest, M., Dahlan, W., & Keeratipibul, S. (2022). Modern on-Site Tool for Monitoring Contamination of Halal Meat with Products from Five Non-Halal Animals Using Multiplex Polymerase Chain Reaction Coupled with DNA Strip. *Food Control*, *132*, 108540. https://doi.org/10.1016/j.foodcont.2021.108540

Esteki, M., Regueiro, J., & Simal-Gándara, J. (2019). Tackling Fraudsters with Global Strategies to Expose Fraud in the Food Chain. *Comprehensive Reviews in Food Science and Food Safety*, *18*(2), 425–440. https://doi.org/10.1111/1541-4337.12419

Halimi, F. F., Gabarre, S., Rahi, S., Al-Gasawneh, J. A., & Ngah, A. H. (2021). Modelling Muslims' Revisit Intention of Non-Halal Certified Restaurants in Malaysia. *Journal of Islamic Marketing*, 1–25. https://doi.org/10.1108/JIMA-01-2021-0014

Hasibuan, F. E. B., Mantiri, F. R., & Rumende, R. R. . (2017). Kajian Variasi Sekunes Intraspesies dan Filogenetik Monyet Hitam Sulawesi (*Macaca nigra*) dengan Menggunakan Gen CO1. *Jurnal Ilmiah Sains*, *17*(1), 59–67. https://doi.org/10.35799/jis.17.1.2017.15558

Huq, A. K. O., Uddin, I., Ahmed, E., Siddique, M. A. B., Zaher, M. A., & Nigar, S. (2022). Fats and Oils Adulteration: Present Scenario and Rapid Detection Techniques. *Food Research*, *6*(1), 5–11. https://doi.org/10.26656/fr.2017.6(1).116

Kalendar, R., Khassenov, B., Ramankulov, Y., Samuilova, O., & Ivanov, K. I. (2017). FastPCR: An in silico tool for fast primer and probe design and advanced sequence analysis. *Genomics*, *109*(3–4), 312–319. https://doi.org/10.1016/j.ygeno.2017.05.005

Kang, T. S., & Tanaka, T. (2018). Comparison of Quantitative Methods Based on SYBR Green Real-Time qPCR to Estimate Pork Meat Adulteration in Processed Beef Products. *Food Chemistry*, *269*, 549–558. https://doi.org/10.1016/j.foodchem.2018.06.141

Kusnadi, J., Ashari, N. A., & Arumingtyas, E. L. (2020). Specificity of Various Mitochondrial DNA (mtDNA), ND5, D-Loop, and *Cyt-b* DNA Primers in Detecting Pig (*Sus scrofa*) DNA Fragments. *American Journal of Molecular Biology*, *10*(03), 141–147. https://doi.org/10.4236/ajmb.2020.103010

Luo, A., Zhang, A., Ho, S. Y. W., Xu, W., Zhang, Y., Shi, W., Cameron, S. L., & Zhu, C. (2011). Potential Efficacy of Mitochondrial Genes for Animal DNA Barcoding: A Case Study Using Eutherian Mammals. *BMC Genomics*, *12*(84), 1–13. https://doi.org/10.1186/1471-2164-12-84

Manalu, H. Y., Sismindari, S., & Rohman, A. (2019). The Use of Primer-Specific Targeting on Mitochondrial *Cytochrome b* Combined with Real-Time Polymerase Chain Reaction for the Analysis of Dog Meat in Meatballs. *Tropical Life Sciences Research*, *30*(3), 1–14.

https://doi.org/10.21315/tlsr2019.30.3.1

Martín, I., García, T., Fajardo, V., Rojas, M., Hernández, P. E., González, I., & Martín, R. (2007). Technical Note: Detection of Cat, Dog, and Rat or Mouse Tissues in Food and Animal Feed Using Species-specific Polymerase Chain Reaction. *Journal of Animal Science*, *85*(10), 2734–2739. https://doi.org/10.2527/jas.2007-0048

Ni'mah, A., Kartikasari, Y., Pratama, A. D., Kartikasari, L. R., Hertanto, B. S., & Cahyadi, M. (2016). Detection of Pork Contamination in Fresh and Cooked Beef Using Genetic Marker Mitochondrial-DNA *Cytochrome b* by Duplex-PCR. *Journal of the Indonesian Tropical Animal Agriculture*, *41*(1), 7–12. https://doi.org/10.14710/jitaa.41.1.7-12

Nugroho, K., Terryana, R. T., & Lestari, P. (2017). Metode Ekstraksi DNA Cabai (*Capsicum annuum* L.) Menggunakan Modifikasi Buffer CTAB (Cethyl Trimethyl Ammonium Bromide) Tanpa Nitrogen Cair. *Scripta Biologica*, *4*(2), 91–94. https://doi.org/10.20884/1.sb.2017.4.2.423

Nuraini, H., Primasari, A., Andreas, E., & Sumantri, C. (2012). The Use of *Cytochrome b* Gene as a Specific Marker of the Rat Meat (*Rattus norvegicus*) on Meat and Meat Products. *Media Peternakan*, *35*(1), 15–20. https://doi.org/10.5398/medpet.2012.35.1.15

Piskata, Z., Pospisilova, E., & Borilova, G. (2017). Comparative Study of DNA Extraction Methods from Fresh and Processed Yellowfin Tuna Muscle Tissue. *International Journal of Food Properties*, *20*(1), S430–S443. https://doi.org/10.1080/10942912.2017.1297953

Prachugsorn, A., Thanakiatkrai, P., Phooplub, K., Ouiganon, S., Sriaead, Y., Thavarungkul, P., Kanatharana, P., Buranachai, C., & Kitpipit, T. (2022). Detection of Porcine DNA in Food Using Direct Asymmetric PCR and Catalyzed Hairpin Assembly Fluorescent Biosensor: A Novel Assay for Halal Food Analysis. *Food Control*, *139*, 108989. https://doi.org/10.1016/j.foodcont.2022.108989

Priyanka, V., Ramesha, A., Gayathri, D., & Vasudha, M. (2021). Molecular Characterization of Non-Biogenic Amines Producing *Lactobacillus plantarum* GP11 Isolated From Traditional Pickles Using HRESI-MS Analysis. *Journal of Food Science and Technology*, *58*(6), 2216–2226. https://doi.org/10.1007/s13197-020-04732-8

Raharjo, T. J., Nuryanti, I., Patria, F. P., & Swasono, R. T. (2018). Mitochondrial ND-1 Gene-Specific Primer Polymerase Chain Reaction to Determine Mice Contamination in Meatball. *International Food Research Journal*, *25*(2), 638–642. Retrieved from http://www.ifrj.upm.edu.my/25%20(02)%202018/(26).pdf

Salahudin, A., Ramli, M. A., Zulkepli, M. I. S., & Razak, M. I. A. (2018). Issues in Halal Meat Product and Authentication Technology from Islamic Perspectives. *International Journal of Academic Research in Business and Social Sciences*, *7*(12), 1305–1315. https://doi.org/10.6007/IJARBSS/v7-i12/3768

Saputra, I., Jaswir, I., & Akmeliawati, R. (2018). Identification of Pig Adulterant in Mixture of Fat Samples and Selected Foods based on FTIR-PCA Wavelength Biomarker Profile. *International Journal on Advanced Science, Engineering and Information Technology*, *8*(6), 2341. https://doi.org/10.18517/ijaseit.8.6.7689

Sasmito, D. E. K., Kurniawan, R., Muhimmah, I., & Metode, M. (2014). Karakteristik Primer pada Polymerase Chain Reaction (PCR) untuk Sekuensing DNA : Mini Review. *Seminar Nasional Informatika Medis*, 93–102.

Sharma, A., Batra, N., Garg, A., & Saxena, A. (2017). Food Adulteration: A Review. *International Journal for Research in Applied Science & Engineering Technology (IJRASET)*, *5*(11), 686–689.

Tibola, C. S., da Silva, S. A., Dossa, A. A., & Patrício, D. I. (2018). Economically Motivated Food Fraud and Adulteration in Brazil: Incidents and Alternatives to Minimize Occurrence. *Journal of Food Science*, *83*(8), 2028–2038. https://doi.org/10.1111/1750-3841.14279

Vaithiyanathan, S., Vishnuraj, M. R., Narender Reddy, G., & Srinivas, C. (2021). Authentication of Camel Meat Using Species-Specific PCR and PCR-RFLP. *Journal of Food Science and Technology*, *58*(10), 3882–3889. https://doi.org/10.1007/s13197-020-04849-w

Wirdateti, Indriana, E., & Handayani. (2016). Analisis Sekuen DNA Mitokondria Cytochrome Oxidase I (COI) mtDNA Pada Kukang Indonesia (*Nycticebus* spp.) sebagai Penanda Guna Pengembangan Identifikasi Spesies. *Jurnal Biologi Indonesia*, *12*(1), 119–128. https://doi.org/10.14203/jbi.v12i1.2322

Yalçınkaya, B., Yumbul, E., Mozioğlu, E., & Akgoz, M. (2017). Comparison of DNA Extraction Methods for Meat Analysis. *Food Chemistry*, *221*, 1253–1257. https://doi.org/10.1016/j.foodchem.2016.11.032

Zhang, J., Xu, Y., Ling, X., Zhou, Y., Lin, Z., Huang, Z., Guan, H., Xiao, Y., Xu, W., & Kan, B. (2020). Identification of Diarrheagenic *Escherichia coli* by A New Multiplex PCR Assay and Capillary Electrophoresis. *Molecular and Cellular Probes*, *49*, 101477. https://doi.org/10.1016/j.mcp.2019.101477