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FULL PAPER

A simplex and multiplex PCR assay for simultaneous detection of beef, pork, and chicken meat in sausages based on mitochondrial DNA *Cytochrome oxidase sub-unit I*

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Abstract

Adulteration in the processed beef product industry has long been an issue affecting consumers' rights and legal protection as beef is an expensive raw material. The intense market competition opens up opportunities for manufacturers of processed beef products to counterfeit and mixes their products with other cheap meat ingredients such as chicken and pork. In addition, the use of pork to substitute beef in the production of sausages without proper labelling on the final products is both an adulteration and halal assurance issue in the Muslim-majority market. Multiplex PCR is a sensitive assay used for the identification of raw materials used in the production of processed food and it reduces the cost and time of analyzing a large number of samples. The purpose of this research was to apply the multiplex PCR assay targeting mitochondrial DNA *Cytochrome Oxidase Sub-Unit I* (*COI*) sequence and to determine the detection limits for DNA concentrations in beef, pork, and chicken. The DNA genomes that were extracted from each meat were diluted to concentrations of 25; 10; 1; 0.1; 0.01; and 0.001 ng/µL, and then used as a DNA template for the simplex- and multiplex-PCR. The results of simplex- and multiplex-PCR showed that the *COI* primers used were able to amplify the DNA of bovine, porcine, and chicken accurately as indicated by amplicons of 263 bp, 168 bp, and 596 bp, respectively. It was concluded that the simplex- and multiplex-PCR methods using *COI* primers can be used to identify bovine, porcine, and chicken DNA isolated from sausages with a sensitivity of 0.001 ng/µL.

1. Introduction

Meat is a protein-rich food ingredient containing about 15-22% of protein and a complete amino acid composition (Orkusz, 2021). Processed beef products are popular as most of them are acceptable in taste and contain the nutrients needed for fulfilling daily nutrient intake, particularly essential amino acids and iron (Gómez *et al*., 2020). The increase in market competitiveness results in the occurrence of fraud among manufacturers. The fraud includes the substitution of expensive raw materials in processed meat products with cheap ones from other sources, e.g., substituting beef with chicken or pork in processed beef products (Unajak *et al*., 2011; Doosti *et al*., 2014). The act of meat adulteration may change the halal status of meat and cause health hazards due to allergenic substances (Cahyadi *et al*., 2020; Khikmawati *et al*., 2021). Therefore, halal assurance of processed meat products is necessary and must be upheld to provide a sense of security and trust to Muslim consumers.

Food authenticity is a key factor in improving customers' trust in food products. Presently, a molecular technique using DNA is widely used for food authentication. DNA is an informative matter than

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protein and is easy to be extracted even in a small trace of organic stuff (Hellberg and Morrisey, 2011). The DNA-based method has been proven to be specific, sensitive and effective in identifying species in processed food products (Cai *et al*., 2021). Polymerase chain reaction (PCR) is a DNA-based assay commonly used to identify the source of raw materials in processed products. Multiplex PCR is among many approaches to optimising PCR techniques to identify animal species in processed meat products. This sensitive method uses more than one primer in a single PCR tube, so it reduces the cost and time of analyzing a large number of samples (Cai *et al*., 2021). Food authentication using this approach depends on the nuclear or mitochondrial markers. Mitochondrial markers, e.g., Cyt b, CO III, and ATPase subunit 8/6, have been studied and widely used as they have a large copy number within a single cell and are more likely stable in the processed products than the nuclear ones (Kumar *et al*., 2015; Qin *et al*., 2019). *Cytochrome oxidase Sub Unit I* (*COI*) is one of the mitochondrial markers in DNA barcoding as it is considered effective in identifying animal species (Costa *et al*., 2007). Wang *et al*. (2018) developed a reliable method for the detection of adulteration in raw and processed buffalo meat products using *COI* as the marker.

It is required to determine the minimum limit of DNA concentration for species identification in meat products using PCR. Besides, the tolerance level for porcine DNA in food must be zero due to religious law. Previously, Khoirinisah *et al*. (2020) designed specific primers targeting *COI* for beef, pork and chicken identification using raw meat as the sample. However, the detection limit and species identification using commercial sausages have not been set in that study. To determine the sensitivity of the PCR test, extracted DNA was diluted in several dilutions (Rosilawati *et al*., 2002). Therefore, this study aimed to apply the multiplex PCR assay using *COI* primers in determining the detection limits for chicken, bovine and porcine DNA isolate concentrations in sausages.

2. Materials and methods

2.1 Sample collection

Beef (*Bos taurus*), chicken (*Gallus gallus domesticus*), pork (*Sus scrofa*), and twenty commercial sausages were obtained from a local market. The samples were then stored at -20°C in the Division of Biology, Integrated Laboratory of Universitas Sebelas Maret, Surakarta.

2.2 DNA extraction

DNA extraction was carried out by using the Salt method (Cawthorn *et al*., 2011). The meat and sausage samples were weighed as much as 50 mg and put into a microtube, mashed by using a micro pestle and dissolved in 400 µL of lysis buffer. Next, 40 µL of 20% SDS was added along with 20 µL of proteinase K (20 mg/mL) to the sample and vortexed. The liquid mixture was then incubated using a water bath at 65°C for 60 mins. A total of 300 µL of 6 M NaCl was added to the microtube and mixed slowly. The sample was then vortexed for 30 s and centrifuged for 30 mins at a speed of 10,000 rpm (HETTICH ZENTRIFUGEN MIKRO 22 R, Andreas Hettich GmbH and Co. KG, Germany). The middle layer was transferred to a new tube, added with isopropanol with a ratio of 1:1, and vortexed. Samples were incubated at -20°C for 60 mins, then centrifuged at 10,000 rpm for 20 mins. The supernatant was removed, and 1 mL of 70% ethanol was added. Samples were centrifuged at 10,000 rpm for 5 mins. The supernatant was discarded. Then, the samples were dried, and 50 µL of TE buffer was added. After the extraction process was complete, electrophoresis was carried out on the samples using 1% agarose gel and visualized using gel documentation (Glite UV Gel Documentation System, Pacific Image, Taiwan). The quantity of DNA was measured by using a spectrophotometer (Implen Nanophotometer 190-1100 NM, Implen GmbH, Munich, Germany). DNA concentration (ng/µL) and DNA purity in optical density (OD) were measured at a wavelength of 260/280. The sample dilution was carried out on the extracted liquid for the samples of beef, pork, and chicken with initial concentrations of 327 ng/µL, 288 ng/µL, and 574 ng/µL, respectively. The diluted samples were then re-diluted several times to produce the following concentrations: 25; 10; 1; 0.1; 0.01; and 0.001 ng/µL

2.3 Simplex and multiplex PCR

The amplification of target DNA was performed according to the method of Khoirinisah *et al*. (2020). The primer sets of the *COI* are presented in Table 1. The PCR reaction was carried out in a PCR thermal cycler machine (GENEAMP® PCR SYSTEM 9700, Thermo Fischer Scientific, Singapore). For multiplex PCR assay, meat samples were prepared from a combination of beef, pork, and chicken. The total volume of reaction in the microtube was 25 µL, consisting of 12.5 µL of TopTaq Master Mix (TOPTAQ MASTER MIX KIT 250, Qiagen GmbH, Hilden, Germany), 2.5 µL of 10x CoralLoad Concentrate (TOPTAQ MASTER MIX KIT 250, Qiagen GmbH, Hilden, Germany), 1 µL of each primer, and 1 µL of template DNA and RNase-free H_2O (TOPTAQ MASTER MIX KIT 250, Qiagen GmbH, Hilden, Germany). The initiation stage was performed at 95°C for 3 mins and continued with 35 denaturation cycles at

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Table 1. Specific primers used for PCR.

Species	Primer (5‘ to 3’)		Amplicons (bp)
Chicken	Forward	TTCTTCGGACACCCCGAAG	596
	Reverse	CTAGGCCCAGGAAATGTT	
Porcine	Forward	TTCTTCGGACACCCCGAAG	168
	Reverse	TGGTGAGCCCATACGATA	
Bovine	Forward	TTCTTCGGACACCCCGAAG	263
	Reverse	CGGTTGGAATAGCAATAA	

95°C for 15 s. The annealing stage was set at 58°C for 30 s, followed by an extension at 72°C for 30 s, and the final extension was set at 72°C for 10 mins. The PCR reaction was repeated for 35 cycles. The results of the reaction were electrophoresed on 2% agarose gel, compared with 100 bp marker ladder DNA to determine the presence of DNA bands of target species, and visualized by using a gel documentation system (Gelview Version 1.0.0.6, Taiwan).

3. Results and discussion

3.1 Simplex PCR for beef

The presence of bovine DNA in the gel document results was indicated by the DNA band length of 263 bp as shown in Figure 1. The visualization results show that the bovine DNA bands were visible at the two highest concentrations (25 and 10 ng/µL). The bands got thinner as DNA concentration was getting lower (1; 0.1; and 0.01 ng/µL). The lowest detection limit (0.001 ng/µL) was also detected. Low DNA concentration can also be caused by the small amount of muscle tissue. Erwanto *et al*. (2014) mentioned that the band visibility was affected by the polymerase enzyme and the annealing temperature used in the PCR process. Bai *et al*. (2009) stated that the detection limit test for bovine species using the common primer multiplex-PCR resulted in a visible minimum detection limit of 0.1 ng/µL. Dai *et al*. (2015) in his research concluded that the minimum detection limit that can be seen in bovine species using *COI* primers was 0.01 ng/µL. This indicates that the method used in this study can detect the presence of bovine DNA more accurately than in previous studies.

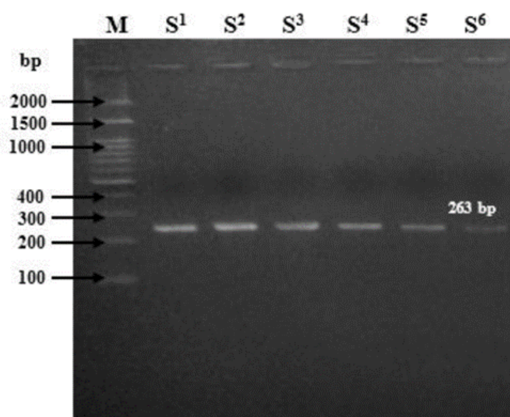

Figure 1. Electrophoregram of simplex PCR sensitivity of *COI* by using beef as samples. M is the 100 bp marker ladder; S1 to S6 are beef samples with the concentrations of 25; 10; 1; 0.1; 0.01; and 0.001 ng/µL respectively.

3.2 Simplex PCR for pork

The presence of porcine DNA in the gel document results was indicated by the DNA band length of 168 bp as shown in Figure 2. The visualization results show that the porcine DNA bands were visible at the three highest concentrations (25; 10; and 1 ng/µL). The bands got thinner as DNA concentration was getting lower (0.1 and 0.01 ng/µL). The lowest detection limit (0.001 ng/µL) was also detected even though the band was very thin. Tanabe *et al*. (2007) reported that the minimum limit of detection seen in porcine DNA by using oligonucleotide primers was 0.001 ng/µL. Another study using species-specific primers targeting the ND5 for porcine DNA found that the visible minimum detection limit was 0.02 ng/µL (Ali *et al*., 2015). This indicates that this study had the same results in detecting the porcine DNA in diluted pork samples.

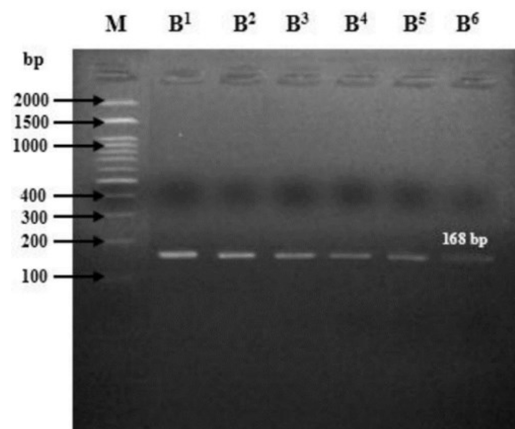

Figure 2. Electrophoregram of simplex PCR sensitivity of *COI* by using pork as samples. M is the 100 bp marker ladder; B1 to B6 are pork samples with concentrations of 25; 10; 1; 0.1; 0.01; and 0.001 ng/µL respectively.

3.3 Simplex PCR for chicken

The presence of chicken DNA in the gel document results was indicated by the DNA band length of 596 bp as indicated in Figure 3. The visualization results show that the DNA bands of the chicken were visible at the two highest concentrations (25 and 10 ng/µL). The DNA bands subsequently faded at lower concentrations (1 and 0.1 ng/µL). However, the bands at the concentration of 0.01 ng/µL were more visible than those at the

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concentration of 0.1 ng/µL. The lowest concentration (0.001 ng/µL) was the lowest detectable limit. Dai *et al*. (2015) reported that the minimum detection limit that could be seen for chicken DNA using *COI* primers was 0.001 ng/µL. This indicates that this study had the same results in detecting the chicken DNA in diluted chicken meat samples.

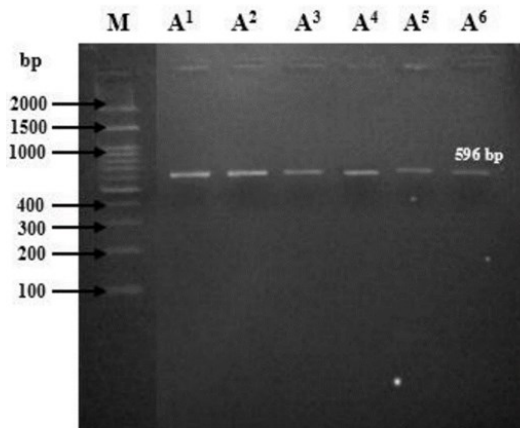

Figure 3. Electrophoregram of simplex PCR sensitivity of *COI* by using chicken as samples. M is the 100 bp marker ladder; A1 to A6 are chicken samples with concentrations of 25; 10; 1; 0.1; 0.01; and 0.001 ng/µL respectively.

3.4 Multiplex PCR

The DNA bands of each DNA concentration are specified in Figure 4. The presence of bovine, porcine, and chicken DNA in the gel document results was indicated by the band lengths of 263 bp, 168 bp, and 596 bp, respectively. The visualization results show that the DNA bands of bovine were the clearest among other species, followed by chicken and porcine. The lightness or darkness of the DNA bands that were produced depended on the quality of the target sequences that were successfully amplified beside the concentration of DNA in the sample. The higher the DNA concentration was, the brighter the resulting DNA band would be (Tenriulo *et al*., 2001). Qin *et al*. (2019) reported that the limit detection of 0.05% for chicken, duck, pork and beef in commercial meat products was determined using other mitochondrial genes, i.e., Cyt b, CO III, and ATPase subunit 8/6. Matsunaga *et al*. (1999) conducted a detection limit test on six different types of meat, e.g., beef, pork, chicken, goat, lamb, and horse and concluded that the detection limit obtained was 0.25 ng/µL. Meanwhile, Wang *et al*. (2018) found that the limit of detection for target DNA was 1 pg. These suggest that the results in this study were in accordance with the

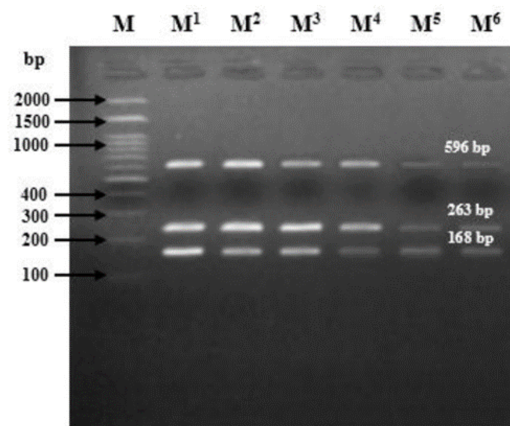

Figure 4. Electrophoregram of multiplex PCR sensitivity of *COI* by using beef, pork, and chicken as samples. M is the 100 bp marker ladder; M1 to M6 are combination samples with the concentrations of 25; 10; 1; 0.1; 0.01; and 0.001 ng/µL respectively.

Table 2. Species identification in commercial sausages sold in retails.

No.	Sample ID	Meat composition on the package	Species identified by multiplex-PCR		
			Bovine	Chicken	Porcine
1	A	Beef	+	+	-
2	B	Beef	+	+	-
3	C	Beef	+	+	-
4	D	Beef	+	+	-
5	E	Beef	+	+	-
6	F	Beef	-	+	-
7	G	Beef	+	+	-
8	H	Beef	+	+	-
9	I	Beef	+	+	-
10	J	Beef, Chicken	+	+	-
11	K	Beef	+	+	-
12	L	Beef	+	+	-
13	M	Beef	+	+	-
14	N	Beef	+	+	-
15	O	Beef	+	+	-
16	P	Beef	+	+	-
17	Q	Beef	+	+	-
18	R	Beef	+	+	-
19	S	Beef	+	+	-
20	T	Beef	+	+	-

+ indicates sample positively containing species; - indicates sample not containing species.

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previous finding by Wang *et al*. (2018), and the method used can be considered a sensitive assay.

The developed technique was demonstrated in commercial beef sausages and the result showed that the multiplex assay was effective. This is approved by the amplification results shown in Figure 5. The multiplex PCR revealed that none of the 20 samples contained pork or was contaminated with porcine DNA (Table 2). However, only one sample mentioning the exact main ingredient (beef and chicken meat) on the label was proved to be appropriate. Therefore, 95% of the beef sausage samples were contaminated with chicken DNA with one sample proved to be fully adulterated. The contamination of chicken DNA in the beef sausages was probably caused by the use of the same processing facilities or the use of chicken meat to substitute some portion of beef. DNA identification using the present approach, however, has a drawback in suggesting the proportion of the ingredient used in the products. Droplet digital PCR (ddPCR) assay has been observed as a promising technique to determine meat proportion in processed meat products based on the DNA concentration (Köppel *et al*., 2019).

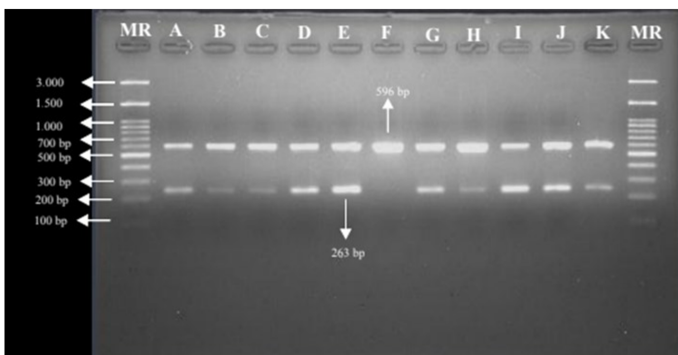

Figure 5. Electrophoregram of multiplex PCR of *COI* by using commercial sausage as samples. MR is 100 bp marker ladder; A to K are commercial sausage samples.

4. Conclusion

The simplex- and multiplex-PCR assays by using *COI* primers as marker can be used to determine the detection limit for meat species identification, i.e., beef, pork, and chicken, with a sensitivity value of 0.001 ng/µL.

Conflict of interest

The authors declare no conflict of interest.

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