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Specificity of Various Mitochondrial DNA (*mt*DNA), *ND*5, *D-Loop*, and *Cty-b* DNA Primers in Detecting Pig (*Sus scrofa*) DNA Fragments

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Abstract

Polymerase Chain Reaction (PCR) is an accurate, simple and fast analytical method. This technique is widely used in the identification of meat adulteration and meat-based processed food products. Three Mitochondrial DNA (mt-DNA) primers NADH Dehydrogenase sub unit 5 (*ND*5), *D-Loop*, and Cytochrome b (*Cyt-b*) were tested for their specificity in detecting of pig (*Sus scrofa*) DNA fragments. DNA genome from 6 meat samples (pork, beef, goat, lamb, and chicken) was amplified by PCR technique using three pairs of primers (*ND*5, *D-Loop*, *and Cyt-b*) and sequenced. The results of amplification using the three primers produced specific DNA bands with the lengths of 232 bp, 951 bp, and 404 bp, respectively. Comparison results with *ND*5, *D-Loop*, and *Cyt-b* gene sequences resulted in similarity values of 100%, 97%, and 99%, respectively. These showed that the mt-DNA primers of *ND*5, *D-Loop*, and *Cyt-b* genes can be recommended as specific primers in detecting pig (*Sus scrofa*) DNA fragments.

Keywords

Cyt-b, *D-Loop*, *mt-DNA*, *ND*5, Pigs, Pork, Specificity

1. Introduction

Pork adulteration in various commercial processed food products is one of the main and crucial problems in the food industry. The presence of adulteration affects food quality, food safety (halal), and health, has the potential to seriously reduce the value of the product [1] [2]. The United States Pharmacopeial Con-

vention (USP) database records 2000 cases of food forgery that occurred in 1980-2012 [3] [4]. In regions or countries with high beef prices such as Korea, Japan, China, etc., often products labeled beef are intensely fraudulent with pork for the benefit of greater economic benefits [2] [5].

Identification of species authenticity in meat samples is needed to provide product clarity and safety for consumers in consuming certain foods [2] [5] [6] [7]. Food authentication is not only focused on efforts to prevent counterfeiting of commercial food, but also food safety is related to the possibility of substance causing allergic and toxic food. Current species detection methods can be performed using protein or DNA analysis. DNA-based analysis methods include quantitative real-time PCR, Restricted Fragment Length Polymorphism PCR (PCR-RFLP) and species-specific qualitative PCR [2] [5].

The Polymerase Chain Reaction (PCR) technique is widely applied for the analysis of meat-based processed food products because it is fast, simple/specific, and sensitive [1] [8] [9] [10]. To support the process of detection of adulteration, many PCR-based methods have been developed using primers designed based on mitochondrial DNA [2] [11]. Mitochondrial DNA (mt-DNA) originates from mitochondrial organelles with nucleotide structures that are similar to their parent and are abundant in cells [12]. The method in the form of species-specific PCR is a method of detecting meat adulteration because of the specificity of the target sequences detected based on DNA sequences [6]. Although the PCR method requires special equipment and reagents that are relatively expensive, it is still more economical than other analysis methods.

Primer specificity is an important determinant of the success of the PCR technique. Another factor that influences is DNA template concentration. Various conventional DNA isolation methods and commercial kits have been widely used. The use of conventional DNA isolation methods is relatively more expensive, requires patience, time-consuming, and uses hazardous chemicals. The use of kits has also been done; however, it tends to produce lower DNA concentrations compared to using conventional methods [13]. Another alternative method of DNA isolation is the Alkaline-lysis method. This method is quite simple, using temperature heating and alkaline treatment for the stages of cell lysis and DNA isolation [10]. In this research, the application of DNA isolation method uses Alkaline-lysis modification and tests the primer specificity of mt-DNA (ND5, D-Loop and Cyt-b) in detecting pig DNA fragments using conventional PCR techniques.

2. Materials and Methods

2.1. Material

Meat samples (beef, goat, lamb and chicken) and pork as a positive control obtained from supermarkets/markets/supermarkets in the city of Malang, East Java, Indonesia. 0.5 M NaOH; 0.01 M EDTA pH 8; 2 M NH_4CH_3COOH; Isopropanol; Ethanol 70%; TE Buffer pH 7.6 and aquadest [10] [14]. Reaction mixture

consists of Go Taq Green Master Mix (PROMEGA); DNA ladder 1 kb; BSA (Bovine Serum Albumin) 10 mg/ml and mt-DNA primers 10 pmol/µl (forward & reverse) [12]. Agarosa gel electrophoresis uses 1.5% agarose, TBE buffer, Ethidium Bromide, Loading Dye, and 1 Kb DNA Ladder [15].

2.2. Methods

Isolation of meat DNA (pork, beef, goat, lamb, and chicken) was done by modifying the Alkali procedure [10]. Quantitative test of DNA isolation results using nano drop spectrophotometer (ND1000). Amplification using PCR technique with 3 types of primers including species-specific mt-DNA primer ND5 (F5'-CAT TCG CCT CAC TCA CAT TAA CC-3' and R5'-AAG AGA GAG TTC TAC TG GGT CTG TAG-3') [1], *Cyt-b* SIM (F5'-GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA-3' and *Cyt-b* Pig (*Sus scrofa*) R5'-GCT GAT AGT AGA TTT GTG ATG ACC GTA-3') [8], and D-Loop (F5'-TAC TTC AGG ACC ATC TCA CC-3' and R5'-TAT TCA GAT TGT GGG CGT AT-3') [9]. The total PCR reaction volume of 10 µl consists of 0.5 µl primer forwards and reverse (10 pmol/µl); 2.75 µl ddH_2O; 5 µl Go Taq Green Master Mix (PROMEGA); 0.25 µl BSA 10 mg/ml; and 1 µl DNA template. The PCR (Takara/Version 3 Model TP600) program consists of hot start at 95˚C (5 minutes), 30 cycles consisting of denaturation at 95˚C (1 minute), annealing at 54˚C (1 minute), extension at 72˚C (1 minute) and final-extension at 72˚C (7 minutes). Electrophoresis using 1.5% agarose gel and visualized using Chemidoc Gel Imaging (Bio-Rad/BR-200). Samples were sequenced at 1st Base Malaysia and analyzed using bioinformatics programs (FinchTV, sequencher 4.1.4 (GeneCode), BLAST (Basic Local Alignment Search Tool)), ClustalX, and Bioedit.

3. Results and Discussion

3.1. DNA Isolation Results Using the Alkali Method

Isolation of meat samples using the alkaline method resulted in DNA concentrations of 126.98 ng/ µl up to 221.65 ng/µl and DNA purity of 1.78 to 2.08 (**Table 1**).

Based on the results of DNA isolation, samples of pork, beef, and lamb are classified as pure from RNA contaminants and proteins with high DNA concentrations. The samples that have contaminated chicken meat contain RNA contaminants and DNA samples of chicken meat (*Gallus domesticus*) contain protein contaminants. Based on the results of the study showed the Alkaline-lysis method is effective for producing DNA with high concentrations and pure protein or RNA contaminants. Alkaline-lysis DNA isolation method is safe, easy, simple, fast, economical, effective, and repeatable so it becomes the recommended method to be applied to meat-based samples. This is supported by previous research, DNA extraction using alkaline methods in chicken blood and tissue samples (*Gallus domesticus*) is simple and fast compared to the modification of the PCI, Kit and conventional methods (PCI) [9].

Table 1. Concentration and purity of DNA isolation results.

Sample	Purity (Å 260/280 nm)	Concentration (ng/µl)
Pork	1.98	132.21
Beef	1.90	143.92
Goat	1.78	194.97
Lamb	1.90	221.65
Chicken	2.08	126.98

3.2. Primer Specificity Test Results

Amplification of pork samples (positive control) using *ND*5 primers produces clear and bright DNA bands with ±250 bp length, using *D-Loop* primers produces DNA bands with ±900 bp length, and using *Cyt-b* primers produces DNA bands with length ± 400 bp (**Figure 1**). The amplification results using the *ND*5 gene primer, *D-Loop*, and *Cyt-b* in beef, goat, lamb, and chicken samples (negative control) did not produce DNA bands (**Figure 1**). This shows that the primers of *ND*5, *D-Loop*, and *Cyt-b* genes are specific in detecting pig DNA fragments. Primers specificity is evidenced by only one size of DNA band produced from the target species (pig).

In a previous study the analysis using the multiplex PCR method succeeded in identifying 6 samples of meat (goat, chicken, beef, lamb, pork, and horse) at the same time, quickly, easily, and sensitive. Specific pig (*Sus scrofa*) *Cyt-b* primers that were designed and amplified using Multiplex PCR produced a DNA band length of 398 bp [8]. The success of the PCR technique is greatly influenced by the primer design. Species-specific *Cyt-b* primers have been tested using multiplex PCR [8] until in this study conventional PCR proved that primers are specific in detecting pork DNA fragments. Conventional PCR techniques generally produce qualitative results for species identification [5].

Other studies of species-specific design primer of *ND*5 are specific and sensitive in detecting pig DNA fragments of 227 bp in length [1]. In addition, the use of mt-DNA *D-Loop* is motivated by the following reasons: *D-Loop* gene is found in mitochondrial DNA that is conserved in many animal species, is stable to heating, and can be used to detect lard [16]. In this study, the three primers are specific in detecting pork/pig DNA fragments.

3.3. Analysis of the Sequence DNA

The amplification results using primer *ND*5, *D-Loop*, and *Cyt-b* genes were further analyzed using a bioinformatics program to produce target DNA sequences (**Figure 2**).

Furthermore, DNA sequences were analyzed using the BLAST program compared to DNA sequences that have been published in Gene Bank, resulting in similarity/ident values and query coverage. Comparative gene sequences from *Sus scrofa* isolate TP mitochondrion (accession code MG 837549) are used to align the DNA primer sequence of *ND*5 and *Cyt-b* genes. Meanwhile, pig (*Sus*

scrofa) isolate *SX*40 and *tRNA-Phe* gene sequences, complete sequence; mitochondrial (accession code MH 430213) is used for comparison of *D-Loop* sequences (Table 2).

Based on the results of the BLAST analysis, the sequences of the *ND*5 gene amplification primers have a 100% compatibility, the *Cyt-b* gene primers have a 99% match, and the *D-Loop* gene primers have a 97% match with each comparison. The results showed the *ND*5 gene primer was the highest and specific match primer in detecting pig DNA fragments compared to the *Cyt-b* and *D-Loop* gene primers. The *ND*5 gene primer has the shortest target DNA sequence length of 232 making the *ND*5 primer effective enough to amplify DNA [17].

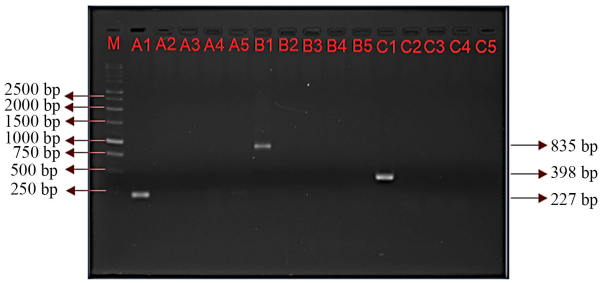

Figure 1. Amplicon produced by A = *ND*5, B = *D-Loop*, and C = *Cyt b* primers. 1 = Pork, 2 = Beef, 3 = Goat, 4 = Chickens and 5 = Lamb.

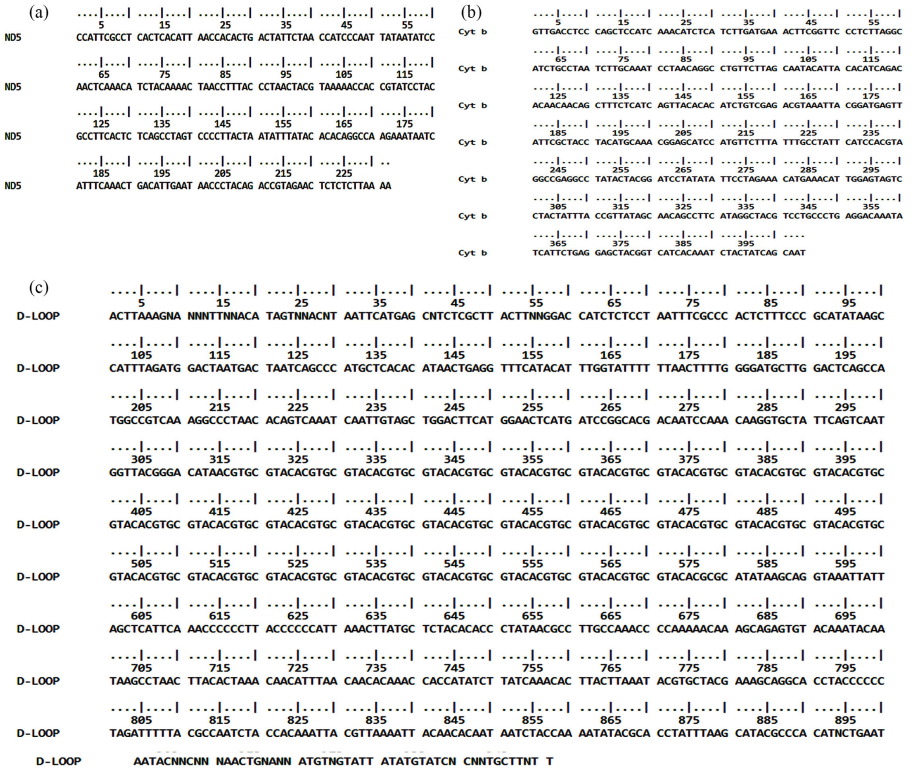

Figure 2. Sequence of *mt-DNA* gene of pork. Description: (a) Amplicon produced by *ND*5 gene primer; (b) Amplicon produced by *D-Loop* gene primer; (c) Amplicon produced by *Cyt-b* gene primer.

Table 2. Similarity analysis of the sequence of DNA using BLAST.

Primers	Sequence length	References	Similarity/Ident.	*Query Cover*	Nucleotides Comparison
ND5	232	MG 837549	100%	98%	229/229
D-Loop	951	MH 430213.	97%	89%	397/400
Cyt b	404	MG 837549	99%	99%	833/855

4. Conclusion

The results of DNA isolation using the Alkali method proved to be effective in producing DNA with high concentrations, relatively free from contaminants, and can be amplified by PCR technique. The three primers from *ND5*, *D-Loop*, and *Cyt-b* genes are specific to detect pig (*Sus scrofa*) DNA fragments of 232 bp, 404 bp, and 951 bp length, respectively. *ND5*, *Cyt-b*, and *D-Loop* genes primers produce amplicons sequences similarity of 100%, 99%, and 97%, respectively. Based on specificity results and sequence confirmation, the *ND5*, *D-Loop*, and *Cyt-b* gene primers are recommended to detect pig DNA fragment.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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