Optimization of Multiplex PCR for Detection of Pork-Contaminated Beef-Based Food Product

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Abstract. The development of globalization affects Indonesia, especially science and technology in the field of food biotechnology. Molecular analysis method, namely Polymerase Chain Reaction (PCR) can be used to detect the food authentication. One of the modifications developed from the conventional PCR method is multiplex PCR which can amplify more than one target gene simultaneously in one PCR reaction. In order to develop an efficient and effective in-house method in detecting food product falsification, such as pork-contaminated beef-based food product, this research aims to obtain the optimum multiplex PCR conditions to amplify the ND5 gene of pork and beef simultaneously by optimizing variations in the primer concentration of pork ND5 primer pairs (Sus-F and Sus-R), beef ND5 primers (Bos-F and Bos-R) and the PCR program. The results of this study showed that the use of 0.5 μM pairs of Sus primers and 0.3 μM pairs of Bos primers in the PCR reaction and the use of the multiplex PCR program at annealing temperatures of 58.7 °C and 48 °C were able to detect mixing of pork DNA and beef simultaneously, according to the size of amplicon for pork was 467 bp and for beef was 206 bp.

Keywords: Multiplex PCR · Food Authentication · ND5 · Sus scrofa · Bos taurus

1 Introduction

The development of globalization affects Indonesia, especially science and technology in the field of food biotechnology. Along with the large number of Muslim consumers in Indonesia, making the Indonesian market a very large Muslim consumer [4]. Food and beverage products circulating in the community do not necessarily provide a sense of security, comfort, tranquility and are suitable for consumption by Muslim consumers, because in Islamic law, it is obligatory for Muslims to consume food and drinks in accordance with Islamic law [12]. In Islam there are already rules and requirements for food, apart from taste, nutrition, cleanliness, safety for consumption, as well as other aspects, namely the status of halal and haram in food and beverages [13]. Food and food ingredients that are considered haram for consumption by Muslims can be classified into four types, namely: carrion, pork and its derivatives, alcohol and its derivatives and blood and its derivatives [13].

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In food products that use meat-based ingredients, including processed beef products, there were many falsifications in the composition of the ingredients used, there were findings regarding beef contaminated with pork [7] Beef contamination occurs due to the increasing demand for beef in the community and the lack of meat supplies in the market, so that the price of beef becomes more expensive and makes the price of pork much cheaper, making it easy for counterfeiting to occur due to the similarity of the physical form of beef and pork which tends to be difficult to be differentiated by consumers [1]. The PCR molecular analysis method was successful in detecting pork in animal products such as sausages, meatballs, skin crackers, and nuggets in Riau Province [10]. PCR method also succeeded in detecting the presence of pork and beef contamination in chicken sausage samples in traditional markets and supermarkets in Italy [3].

One of the advantages of conventional PCR technique is that it can perform detection tests on raw samples, or products that have been subjected to high temperature heating, with low mixing rates. There will be problems if there is adulteration of more than one type of animal, for example in the adulteration and mixing of beef and pork, or beef with rat meat [9]. One of the modifications of the PCR technique is multiplex PCR, which can increase efficiency in the simultaneous analysis of animal species. In addition, the Multiplex PCR method can reduce costs and processing time because it can amplify more than one target gene simultaneously in one PCR reaction [2]. This multiplex PCR method is accurate, economical and fast in detecting animal species from meat ingredients [8]. To get accurate results, the multiplex PCR technique needs to be optimized. Optimization of this concentration is important because it can affect the sensitivity of the PCR reaction, so there can be a risk of false negative results [11].

This research is a continuation of the research of Chatri *et al.* (unpublished) and Mardhotillah *et al.* (2021) who developed an in-house method for the detection of pig genes in food products. Chatri *et al.* have succeeded in designing specific primers for pork (Sus-F and Sus-R) and beef (Bos-F and Bos-R) which amplify a mitochondrial gene of *ND5* (NADH Dehydrogenase Subunit 5), while Mardhotillah *et al.* have succeeded in obtaining optimum PCR conditions for pork primer pairs (Sus-F and Sus-R). In order to develop an efficient and effective in-house method in detecting food product falsification, especially pork-contaminated beef-based food product, this research aims to obtain the optimum multiplex PCR conditions to amplify the *ND5* gene of pork and beef simultaneously.

2 Materials and Methods

This study was a descriptive research by optimizing multiplex PCR condition and was carried out in the Laboratory of Genetics and Biotechnology, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang.

2.1 Materials

The materials used for DNA isolation were fresh beef and pork, phenol (Ribozol), chloroform, absolute ethanol, 75% ethanol, sodium citrate 0.1 M in 10% EtOH, 8 mM NaOH (pH 9), and 1x TE buffer. The materials used for PCR were MyTaq HS Red Mix

Bioline PCR Master Mix, forward primer (Sus-F) and reverse primer (Sus-R), forward primer (Bos-F) and reverse primer (Bos-R), nuclease-free water. The materials used for electrophoresis were agarose powder, Tris-acetate-EDTA (TAE) buffer, GelRed, DNA Ladder 100 bp, and loading dye.

2.2 Methods

2.2.1 DNA Isolation

The samples to be isolated were beef and pork. DNA isolation of these samples were done separately. To perform DNA isolation, 50 mg of the weigh the sample was put into a sterile-1.5 ml microtube. Furthermore, in the lysis stage, 200 μL phenol (Ribozol) reagents were taken and put into a microtube containing the sample, and the sample was crushed using a sterile micropestle, then 800 μL phenol (Ribozol) was added. Sample was incubated for 10 min at room temperature, and the 200 μL chloroform was added. Subsequently, it was centrifuged at 12000 rpm for 15 min at 4 °C, then aqueous phase that was formed was discarded. At the DNA deposition stage, 300 μL of absolute ethanol was added to the sample, then incubated for 3 min, and then centrifuged at 2000 rpm for 5 min at 4 °C. The supernatant was discarded, then in the washing step 1 ml of 0.1 M ($Na_3C6H5O7$) 0,1 M in 10% EtOH was added. The sample was incubated for approximately 30 min at room temperature (microtube was homogenized once every 5 min), and centrifuged at 2000 rpm for 5 min at 4 °C then the supernatant was discarded. The DNA washing step was repeated again, then 1 ml of 75% ethanol was added and the microtube was inverted. Then the sample was incubated for approximately 15 min at room temperature. And samples were centrifuged at 2,000 rpm for 5 min at 4 °C. The next step is to redissolve the DNA pellet, the supernatant was removed and the DNA pellet was dried for 10 min. Then the pellet was added with 50 μL NaOH (pH 9) and centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant was transferred to a new microtube. DNA sample was measured using a nanospectrophotometer to analyze itsquality and quantity. A good purity of DNA was the one with A260/280 ratio of 1.8–2.0.

2.2.2 Optimization of Multiplex PCR Conditions

The PCR reaction for *ND5* gene amplification of pork and beef was carried out simultaneously in one PCR tube. Optimization was done by differentiating the concentration of the forward and reverse primers (Table 1). The composition of the PCR reaction with a total volume of 20 μL consists of 10 μL of 2× My T.

aq Red Mix Bioline PCR Master Mix, 100 ng of genomic DNA (positive control pork DNA and beef DNA) and 0.2–0.6 μM pork ND5 primers (Sus-F and Sus-R) and 0.2–0.6 μM beef ND5 primers (Bos-F and Bos-R). For the PCR reaction, the volume was made up to 20 by adding nuclease-free water.

The PCR program start with initial denaturation at 94 °C for 2 min, followed by 20 cycles targeting the pork ND5 gene consist of denaturation at 98 °C for 10 s, annealing at 58.7 °C for 30 s, and extension at 72 °C for 45 s. The second step was followed by 20 cycles targeting the beef ND5 gene consist of denaturation at 98 °C for 10 s, annealing at 48 °C for 30 s, and extension at 68°C for 45 s. The PCR process ended with the final

Table 1. Variation of Multiplex PCR Primer Concentration

Primer	Variation of primer concentration (μM)				
	A	B	C	D	E
Bos-F	0,2	0,3	0,4	0,5	0,6
Bos-R	0,2	0,3	0,4	0,5	0,6
Sus-F	0,6	0,5	0,4	0,3	0,2
Sus-R	0,6	0,5	0,4	0,3	0,2

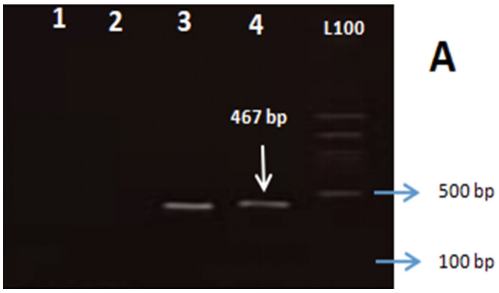

Fig. 1. Electropherogram of multiplex PCR product with primer concentration of A variation.

extension at 68 °C for 7 min. Furthermore, the amplification results were visualized by electrophoresis on 1.5% agarose gel at a voltage of 100 V for 30 min which was stained using GelRed, and using DNA ladder 100 bp as DNA size marker and then observed using GelDoc.

3 Results and Discussion

Optimization of the primer concentration was important because if the concentration used was not appropriate, it can lead to the binding of non-specific primers resulting in false negative or positive results and reducing the sensitivity of the PCR reaction [5]. The amplicon size of Sus primer pair was 467 bp, while the amplicon size of Bos primer pair was 206 bp.

Based on the results of the study of the concentration variations used, it shows that the most optimum primer concentration was the D variation by using 0.5 μM pairs of Bos primers and 0.3 μM pairs of Sus primers in the PCR reaction. PCR product with DNA template used 1). Nuclease-free water as negative control 2). Beef DNA 3). Pork DNA 4). Mix of beef and pork DNA as positive control. The results are shown in "Figs. 1, 2, 3, 4 and 5", the presence of 2 thick DNA bands and according to the size of the target gene with the length of the pork ND5 DNA amplicon 467 bp and for beef ND5 DNA 206 bp [6].

Beside the primer concentration, the program of multiplex PCR that was set on the thermocycler also very important to get the accurate result. The multiplex PCR program

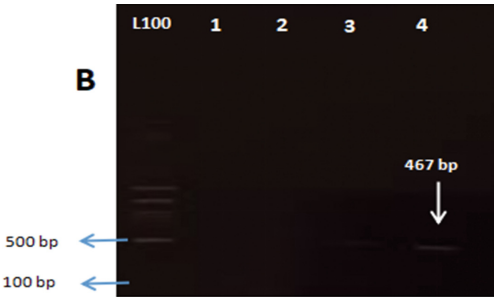

Fig. 2. Electropherogram of multiplex PCR product with primer concentration of B variation.

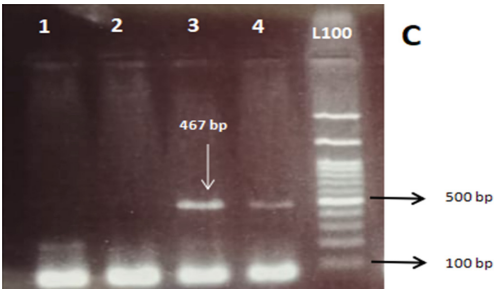

Fig. 3. Electropherogram of multiplex PCR product with primer concentration of C variation.

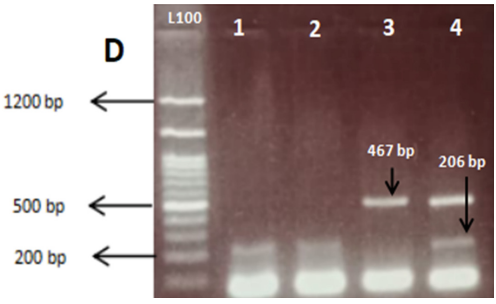

Fig. 4. Electropherogram of multiplex PCR product with primer concentration of D variation.

used two different annealing temperatures, this was done because multiplex PCR used two different pairs of primers, namely beef ND5 primers (Bos-F and Bos-R) and pork ND5 primers (Sus-F and Sus-R). The optimum annealing temperature of each pair of primer were 58.7 °C and 48 °C for pork and beef [6]. The annealing temperature is the right temperature for the primer to attach to the target DNA. If the annealing temperature is too low than the optimum temperature, it will cause false priming, and if the annealing temperature is too high than the optimum temperature, the primer cannot anneal to the target DNA [5]. So that in this study.

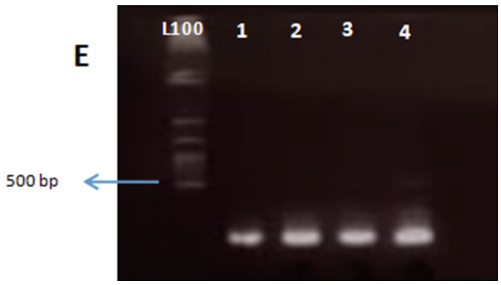

Fig. 5. Electropherogram of multiplex PCR product with primer concentration of E variation. DNA template used as follow: 1). Nuclease-free water as negative control 2). Beef DNA 3). Pork DNA 4). Mix of beef and pork DNA as positive control.

4 Conclusion

By using the optimum condition in the multiplex PCR including primer concentration and cycling program, the results of the multiplex PCR can simultaneously amplify the ND5 gene of pork and beef according to the size of amplicon.

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