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

Afifatul Achyar(✉), Moralita Chatri, and Lisna Khairiyah S

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang, Padang, Indonesia
afifatul.achyar@fmipa.unp.ac.id

Abstract. Food safety is an important point in consuming food, in order to produce good health for the community. But lately there were many foods circulating in the community that are no longer guaranteed to be safe, namely the existence of counterfeiting and mixing beef with other meats such as rat meat. Rat meat is forbidden for Muslims to consume. In addition, rat can be a reservoir for several disease-causing pathogens in humans. This makes the majority of the Muslim community in Indonesia very worried and in dire need of guarantees for safe and halal food. The purpose of this study was to develop an in-house method for detection of rat meat-contaminated beef-based food product using the multiplex PCR method. Multiplex PCR in this study was optimized on the three variation of PCR program. The optimum multiplex PCR conditions to amplify the ND5 gene of rat and beef simultaneously was using the combination of cycling program of Mur-MA primer pair and Bos primer pair that resulting 919 bp and 206 bp DNA bands which were amplicons of therat and bovine ND5 gene, respectively.

Keywords: PCR multiplex · food authentication · ND5 · Bos taurus

1 Introduction

Food is a basic human need that is very important to improve health, so that humans can carry out their daily activities properly. Therefore, food safety becomes very important so that there are no unwanted health disorders. But lately there are many foods circulating in the community that are no longer guaranteed safety [1]. Currently the government stipulates that food rules and processed products of animal origin circulating in the community must meet the requirements of ASUH which is safe, healthy, whole and halal, due to the rampant cases of counterfeiting and mixing of beef with other meats today caused by the increasing price of beef [2].

Food safety is closely related to halal and thayyib in Islam. Halal food is food that is permissible for a Muslim to eat as long as it does not contradict the teachings of the Qur'an and Hadith. Food can be said to be halal if there are no elements or ingredients that are forbidden to eat [3]. Meanwhile, thayyib means good for the body, does not feel sick or other problems arise after consuming it [4].

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.One of the foodstuffs that are not halal in Islamic law other than pigs and dogs is rats [5]. Rat meat is physically difficult to distinguish from beef, especially if it has been through the grinding and cooking process [6]. So the right method is needed to detect the presence or absence of rat meat contamination in beef-based food products. The method that capable in detecting the type of meat used in the processed foods is Multiplex Polymerase Chain Reaction (multiplex PCR). This method is a modification of the conventional PCR method that uses multiple sets of primers simultaneously in amplifying multiple target areas in a single reaction [7].Previous study by Masnaini (2021) had succeeded in designing a primer that can amplify the mitochondrial *ND5* gene in rat and obtained the optimum PCR conditions for the primer pair. Mitochondrial ND5 gene was chosen as the target gene because mitochondrial DNA has multiple copy in each cell. So that the probability to detect the rat DNA in processed food will be higher. In order to develop an efficient and effective in-house method in detecting rat meat contamination in beef-based food product, this research aims to obtain the optimum multiplex PCR conditions to amplify the *ND5* gene of rat and beef simultaneously.

2 Materials and Methods

The research was conducted at the Laboratory of Genetics and Biotechnology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang.

2.1 Materials

The main research materials were beef and meat from rat that caught from landfills. The materials needed for DNA isolation were: Ribozol (phenol), sodium citrate, chloroform, 8mM NaOH, 75% ethanol, absolute ethanol, TE buffer. The materials used in DNA amplification are nuclease-free water, 2x My Taq HS Red Mix, DNA sample, forward primer and reverse primer for rat (Mur_MA_Fwd and Mur_MA_Rev) and bovine (Bos-F and Bos-R) *ND5* gene amplification. The materials used for electrophoresis are agarose, 1x TAE buffer, 100 bp DNA ladder, loading dye, and GelRed.

2.2 Methods

a) DNA Isolation

Samples of rat meat and beef were weighed as much as 50 mg and cut into pieces and each was inserted into a sterile 1.5 ml microtube. At the lysis stage, which was by the addition of 1000 μL Ribozol (phenol) gradually, the first was 200 μL Ribozol pipeted into a microtube containing the sample and then crushed with a sterile micropestle until homogenized. Then 800 μL of Ribozol was added and the sample was incubated for 10 minutes at room temperature. Next 200 μl of chloroform was added and shaked by hand for 15 seconds. Then sample was incubated for 3 minutes at room temperature and centrifuged at a speed of 12,000 rpm for 15 minutes at 4ºC. After being centrifuged the aqueous phase was discarded. Next, 0.3 ml of absolute ethanol was added to the sample and incubated for 3 minutes at room

temperature. Then the sample was centrifuged at 2000 rpm for 5 minutes at 4ºC, then the supernatant was discarded. Then another 1 ml 0.1 M sodium citrate in 10% EtOH was added to the sample and incubated for 30 minutes at room temperature and the tube was inverted every 5 minutes. Then the sample was centrifuged at 2000 rpm for 5 minutes at 4ºC. Then the supernatant was discarded again. The DNA washing was repeated by adding 1 ml 0.1 M sodium citrate in 10% EtOH and incubated again for 30 minutes at room temperature and the tube was inverted every 5 minutes. Then the sample was centrifuged with a speed of 2000 rpm for 5 minutes at 4ºC. Then the supernatant was discarded again. 1 ml of 75% ethanol was added and the tube was inverted and then incubated for 15 minutes at room temperature. Then sample was centrifuged again at 2000 rpm for 5 minutes at 4ºC. The supernatant was discarded again and the DNA (pellet) was air-dried for 10 minutes. 50 μl of 8 mM NaOH was added to the DNA pellet and centrifuged at 12,000 rpm for 10 minutes at 4ºC. Then the supernatant was transferred to a new microtube. Then 50 μl of TE buffer pH 8.0 was added. Furthermore, the concentration and purity of the DNA was measured using nanophotometer. The DNA sample was stored at -20ºC freezer.

b) Multiplex PCR Optimization

The primers were rat primers (Mur_MA_Fwd and Mur_MA_Rev) [8] and bovine primers (Bos_F and Bos_R) [9]. Primer Mur_MA_Fwd has a length of 23 bases with a sequence of 5'-GCAGTTCTCTTCATGATAYATAC-3' while primer Mur_MA_Rev has a length of 18 bases with a sequence of 5'-GTTTCAGGCGTTGGTGTT-3' with a PCR product size of 919 bp. The target gene to be amplified is the *ND5* gene in *Rattus rattus.* Meanwhile the target gene of Bos-F and Bos-R is *ND5* gene in *Bos taurus* with amplicon size is 206 bp.

The composition of the multiplex PCR reaction with a total volume of 20 μl consisting of 10 μl 2x My Taq Red Mix Bioline PCR Master Mix, 100 ng genomic DNA (bovine DNA; rat DNA; mix of bovine DNA and rat DNA as positive control; nuclease-free water as negative control) and 0.3 μM rat ND5 primer (Mur -MA-F and Mur-MA-R) and 0.5 μM bovine ND5 primers (Bos-F and Bos-R). For the PCR reaction, the volume was made up to 20 μl by adding nuclease-free water (NFW).

Since the multiplex PCR used more than a pair of primer, so in this study we optimized the PCR program that is suitable to amplify the *ND5* gene of rat and bovine simultaneously. There were three variation PCR program used in this study (Table 1). The "A" PCR program was carried out using the optimum PCR condition for rat primers (Mur_MA_Fwd and Mur_MA_Rev). The "B" PCR program was carried out using the optimum PCR condition for bovine primers (Bos-F and Bos-R) [10]. The "C" PCR program was set up as a combination of "A" and "B" PCR program. The PCR products were visualized by electrophoresis on 1.5% agarose gel stained using GelRed and observed under UV light using a GelDoc (UVITEC).

3 Result and Discussion

Based on the result of "A" variation of PCR program of rat-bovine multiplex PCR in Fig. 1, the electropherogram showed target amplicon on lane 2 (206 bp of bovine ND5

Table 1. Variation of PCR Program of Multiplex PCR

PCR Program	Step	Temperature (^{O}C)	Duration	Cycle
A	Initial Denaturation	94	2 min	1x
	Denaturation	98	10 s	35x
	Annealing	48	30 s	
	Extension	72	45 s	
	Final Extension	72	7 min	1x
B	Initial Denaturation	94	2 min	1x
	Denaturation	98	10 s	35x
	Annealing	48	30 s	
	Extension	68	45 s	
	Final Extension	72	7 min	1x
C	Initial Denaturation	94	2 min	
	Denaturation	98	10 s	20x
	Annealing	48	30 s	
	Extension	72	45 s	
	Denaturation	98	10 s	20x
	Annealing	48	30 s	
	Extension	68	45 s	
	Final Extension	68	7 min	1x

amplicon) and lane 3 (919 bp of rat ND5 amplicon). While on the lane 4 only showed the band of bovine amplicon which was supposed to be both 206 bp and 919 bp. In Fig. 2 that showed the PCR product from "B" variation of PCR program, only 206 bp DNA bands (bovine amplicons) were observed because the PCR program used was the optimum PCR conditions for the Bos primer pair. While in Fig. 3 the electropherogram showed the appropriate band in each lane. In lane 1 (the negative control) there was no target band except the primer dimer (below 100 bp), in lane 2 which used bovine DNA as template DNA there was a 206 bp band (bovine amplicon), in lane 3 which used rat DNA as template DNA there was a 919 bp band (rat amplicon), and in lane 4 (the positive control) which used a mixture of rat and bovine DNA, there were bands measuring 206 bp (bovine amplicons) and 919 bp (rat amplicons).

The temperature in PCR program play an important role in determining the success of the PCR. The rapidly changing of temperature to heat and cool the PCR reaction will denature the double-helix DNA strand to single-strand DNA (denaturation step) and will be paired with the primer on both strand (annealing step), respectively. Then, the DNA polymerase will synthesize the new strand of DNA by elongating the primer, both forward and reverse (elongation step). In general, the initial denaturation step was set up at 94 °C to 98 °C for 1 to 3 min. Initial denaturation longer than 3 min will inactivate

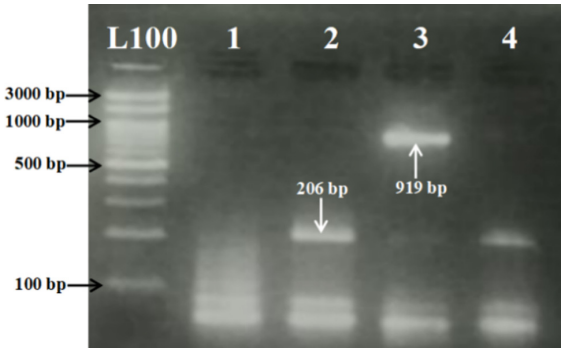

Fig. 1. Electropherogram of multiplex PCR using "A" PCR program. L100 = Geneaid 100 bp DNA Ladder; 1 = nuclease-free water as negative control; 2 = bovine DNA; 3 = rat DNA; 4 = mix of bovine DNA and rat DNA as positive control. ND5 bovine amplicon size is 206 bp and ND5 rat amplicon size is 919 bp.

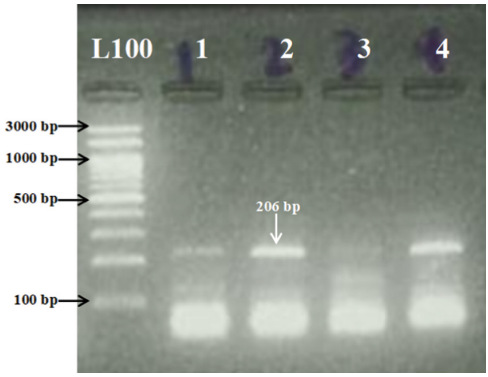

Fig. 2. Electropherogram of multiplex PCR using "B" PCR program. L100 = Geneaid 100 bp DNA Ladder; 1 = nuclease-free water as negative control; 2 = bovine DNA; 3 = rat DNA; 4 = mix of bovine DNA and rat DNA as positive control. ND5 bovine amplicon size is 206 bp.

the DNA polymerase [11]. So that in this experiment, the initial denaturation was set at 94 °C for 2 min.

The annealing temperature is also important for the PCR reaction because the primer annealing process on an already exposed DNA strand requires an optimal temperature. If the temperature is too high it will cause the amplification to fail because there is no primer annealed. Otherwise if the temperature is too low it causes the primer to hybridize to the other side of the genome as a result the DNA formed has low specificity. So it is very important to find the optimum annealing temperature for the amplification process [12].

Generally the elongation or extension temperature is at 70°-72 °C. At this temperature, the Taq DNA polymerase works best on synthesize new strand of DNA according to template DNA. But at some cases such as for AT-rich DNA template, the extention temperature must be reduced below 72 °C, from 60°-68 °C [13]. On the optimum PCR

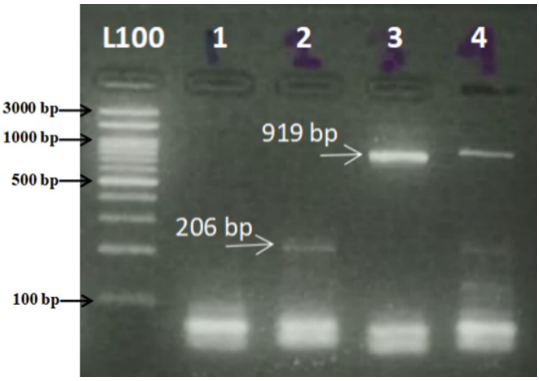

Fig. 3. Electropherogram of multiplex PCR using "C" PCR program. L100 = Geneaid 100 bp DNA Ladder; 1 = nuclease-free water as negative control; 2 = bovine DNA; 3 = rat DNA; 4 = mix of bovine DNA and rat DNA as positive control. ND5 bovine amplicon size is 206 bp and ND5 rat amplicon size is 919 bp.

program ("C") the extension temperature used were 72 °C for a pair of primers mur_MA detecting rat ND5 gene, which was optimized by Masnaini [8]. And 68 °C for a pair of primers Bos detecting bovine ND5 gene which was optimized by Wijaya [10].

4 Conclusion

The optimum multiplex PCR conditions to amplify the *ND5* gene of rat and beef simultaneously was using the combination of cycling program of Mur-MA primer pair and Bos primer pair.

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