Optimization of DNA Isolation on Meat-Based Food Samples Using Phenol-Chloroform Method for the Development of Halal Detection Using In-House Method Analysis

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Abstract. About 85% of Indonesia's population is Moslem. Every Moslem is ordered to eat halal and good food. PCR is the most widely used molecular biology technique for authentication tests, through target DNA analysis, including the detection of pork-derived DNA in food products. DNA isolation is an important step that determines the success of PCR, especially for food samples that have gone through various cooking processes at high temperatures, thus affecting the quantity and quality of DNA isolation results. The phenol-chloroform-based DNA isolation method is a method that's considered capable of producing high-quality DNA although the process is slightly longer than using commercial kits. This study aims to obtain optimum DNA isolation results using the phenol-chloroform method in meat-based food samples. DNA quality was tested pork-specific *ND5* gene PCR Isolation was carried out on pork and meatball samples which were weighed as much as 50 mg and 100 mg. The results showed DNA isolation with a sample weight of 50 mg was better than a sample weight of 100 mg. The DNA concentration at the sample weight of 50 mg and 100 mg were 423.35 ng/µl and 7472.9 ng/µl. DNA purity at the sample of 50 mg and 100 mg were 1.7 and 1.0.

Keywords: DNA Isolation · PCR · ND5 · Phenol-Chloroform

1 Introduction

Indonesia is one of the countries with the largest Muslim population in the world, about 85% of the Indonesian population is Muslim [1]. Every Muslim is commanded to eat "halal food" (i.e.; unforbidden food for consumed by certain criteria) and "thayyib food" (i.e.; good). The House of Representatives (DPR) of the Republic of Indonesia and the President of the Republic of Indonesia has enacted a law on Halal Product Guarantee, abbreviated as JPH, that products that enter, circulate, and are traded in the territory of Indonesia must be certified halal [2]. The obstacle faced by the Muslim community in the consumption of food products is the contamination of food from ingredients that

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are harmful to health, one of which is the mixing of pork and its derivatives [3]. One of the meat-based food products that are popular in Indonesia and are widely found in the market is beef meatballs [4]. Mixing pork in beef meatballs at this time is very common, mixing pork with beef in meatballs has the aim of lowering production prices to be cheaper than using beef alone so that it can generate high profits [1, 5]. Seeing the many cases of fraudulent mixing of pork in processed food products made from meat, of course, makes people nervous, especially those who are Muslim. Therefore, it is necessary to have an effective detection method to identify pork in meat-based processed foods [6].

Polymerase Chain Reaction (PCR) is a molecular biology technique that is widely used for food authentication tests through target DNA analysis, including the detection of pork-derived DNA (pork meat and pork gelatin in food products). The PCR technique is used for halal authentication purposes because of its ability to detect specific targets of DNA sequences in food products or pharmaceutical products [7]. The PCR technique has a sensitive ability to detect the presence of pork in fresh meat and processed products mixed with other ingredients [8].

DNA analysis with PCR is a fast, sensitive, specific, and cheaper alternative for species identification from processed meat-based foods and processed foods that have gone through a long process such as heating which can make DNA difficult to detect. The key to the success of PCR lies in the quality and quantity of DNA used as a template, which depends on the method of DNA isolation of food products. Therefore, an appropriate DNA isolation or extraction method is needed for the detection of species of origin in meat-based processed food ingredients to obtain good quality and quantity of DNA.

DNA extraction is the first stage of molecular research that greatly influences the quality of DNA isolation (Kamaliah, 2017)[9]. Extraction or isolation of DNA is the process of separating DNA from other cell components such as proteins, carbohydrates, fats, and others. DNA extraction consists of three main stages, namely the destruction of cell membranes (lysis), separation of DNA from other cell components, and DNA purification [10].

The DNA extraction process consists of several methods including using physical/mechanical methods by grinding and using *grinding* or *boiling*, as well as using chemical methods with the addition of reagents. One method of extraction or isolation of DNA conventionally is an organic extraction method that can be done by adding organic solutions such as detergent, phenol, and chloroform to lyse cells [11].

In using conventional PCR, it is necessary to develop in-house methods, one of which is optimization [12]. Optimization is required to ensure that the test is as sensitive as required and specific to the desired target. There are several factors that can be changed to get optimal test performance so as to increase sensitivity, specificity, and precision [13].

Test components that are important to be optimized include sample weight for DNA extraction, annealing temperature, PCR sensitivity, primer concentration, and primer specificity. This study aims to optimize the weight of processed meat-based food samples during DNA isolation in order to determine the quality and quantity of DNA isolation

for the feasibility of DNA amplification using the PCR method in detecting pork DNA in meat-based food samples.

2 Material and Methods

2.1 Material

This research was conducted at the Laboratory of Genetics and Biotechnology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang. The ingredients in this study were fresh pork as a positive control and some samples of meatballs for applied tests were taken randomly from several meatball outlets in the city of Padang. For PCR detection of the *ND5* gene in pork, Sus-F and Sus-R primers [14] and MyTaq HS Red Mix, Bioline, and materials for electrophoresis such as agarose and GelRed were used for PCR.

2.2 Methods

2.2.1 Optimization of DNA Isolation in Fresh Meat

DNA isolation was carried out manually using the protocol RiboZol [15]. DNA isolation was carried out separately per sample to avoid contamination between samples. A negative control is done first and positive control is done last. The work area is cleaned first before starting the isolation using 70% alcohol.

To obtain optimum DNA isolation results, namely pure DNA and high concentrations, optimization was carried out with variations in sample mass. The samples (pork) were weighed as much as 50 mg and 100 mg and then each was put into a microtube sterile 1.5 ml. The next step is lysis, pipette 200 μl of phenol (RiboZol), and put into a microtube containing the sample, then the sample was with a ground micropestle sterile. Next, 800 μl of phenol (RiboZol) was added. After the separation step, 50 mg and 100 mg samples were incubated for 10 min at room temperature, then 200 μl of chloroform was added. Then, it was centrifuged at 12,000 rpm for 15 min at 4. After that, the supernatant was discarded. Furthermore, in the DNA deposition stage, the sample was added with 300 μl of absolute ethanol and incubated for 3 min at room temperature, then centrifuged at 2,000 rpm for 5 min at 4 °C. After that, the supernatant was discarded. In the washing stage, the pellet was added with 1 ml 0.1 M sodium citrate/10% ethanol, then incubated the sample for 30 min at room temperature (back and forth in the microtube every 5 min), then centrifuged at 2,000 rpm for 5 min at temperature 4 °C, after which the supernatant was discarded. The washing step was repeated again, then 1 ml of 75% ethanol was added and inverted. Then, the samples were incubated for 15 min at room temperature. Next, the sample was centrifuged at 2000 rpm for 5 min at 4 °C. The next step is to re-dissolve the DNA pellet, the supernatant is removed and the DNA (pellet) is air-dried for 10 min. Then 50 μl of NaOH (pH 9) was added to the pellet, then centrifuged at 12,000 rpm for 10 min at 4 °C. Then the supernatant was transferred to a new microtube, then 50 μl of TE buffer (pH 7–9) was added [15]. Furthermore, the quality and quantity of DNA isolation results were measured with a Nanospectrophotometer. Good DNA purity has an A260/280 ratio of 1.8–2.0.

2.2.2 Isolation of DNA in Meatball Samples

After obtaining optimum DNA isolation conditions, DNA isolation was carried out on meat-based samples, namely meatballs. The working procedure of DNA isolation of meatball samples is the same as optimizing DNA isolation but using the optimum sample mass of 50 mg. Furthermore, the quality and quantity of DNA isolation results were measured with a *Nanospectrophotometer.* Good DNA purity has an A260/280 ratio of 1.8–2.0.

2.2.3 DNA Amplification

Pork-specific primers *(Sus scrofa)* used were primers Sus-F and Sus-R [14] which were designed using software Geneious and NCBI. This primer will amplify the gene in mitochondrial DNA, namely *ND5* (NADH dehydrogenase subunit 5).

The composition of the PCR reaction used was a total volume of 10 μl consisting of 5 μl 2x My Taq HS Red Mix Bioline PCR Master Mix, 1 μl DNA template, 0.4 μM pork-specific forward primer, and 0.4 μM pork-specific reverse primer.. The PCR reaction was made up to 10 μl with the addition of nuclease-free water. PCR was carried out under the following conditions: initial denaturation at 95 for 5 min, followed by 35 cycles consisting of denaturation at 95 °C for 30 s, annealing at 58.7 °C for 30 s, and elongation at 72 °C for 30 s. The PCR process ended with an elongation step at 72 °C for 5 min. Analysis of the presence of DNA seen by electrophoresis.

2.2.4 Analysis of DNA Amplification

Results The PCR results were visualized by electrophoresis of 1.5% agarose gel at a voltage of 100 V for 30 min in 1X TAE buffer. The DNA size marker used was a 100 bp DNA ladder. The PCR and DNA ladder products were added with loading dye and GelRed and then inserted into the agarose gel wells. After that, the electrophoresis device is set the voltage and time, then pressed the button run. Furthermore, the results of the electrophoresis were visualized using the UVITEC gel documentation system and software UVITEC Cambridge Reader.

3 Result and Discussion

In this study, conventional DNA isolation was carried out using phenol-chloroform. DNA isolation is the first step before DNA amplification. The principle of DNA isolation itself is to separate DNA from the components that make up the cell by lysing the cell membrane so that DNA can be removed from the cell. The ratio of the good value of DNA purity A_{260}/A_{280} is 1.8–2.0. Optimization of DNA isolation was carried out in order to find the optimum sample weight to isolate the next DNA sample. The optimum condition of DNA isolation was seen from the concentration value and DNA purity.

The sample weight of 50 mg in porcine DNA (Table 1) had concentration and purity values according to the criteria for good DNA isolation. In the 100 mg boar DNA sample (Table 1) the purity produced was too low, so it did not meet the criteria for amplification. The DNA of meatballs A, B, C, D, and the DNA of beef has a value of A_{260}/A_{280} below 1.8 (Table 1) minus value.

Table 1. Results of Nanophotometer Readings on DNA Isolation Results.

No	Sample	DNA Concetration (ng/μL)	Purity A_{260}/A_{280}
1	DNA of Pork 50 mg	423.35	1.77
2	DNA of Pork 100 mg	7472.9	1.00
3	DNA of Beef 50 mg	7384.1	1.00
4	DNA of Meatball A 50 mg	7386.4	1.00
5	DNA of Meatball A 100 mg	−21.250	1.59
6	DNA of Meatball B 50 mg	7444.4	1.20
7	DNA of Meatball B 100 mg	−29.450	1.51
8	DNA of Meatball C 50 mg	7365.5	1.00
9	DNA of Meatball C 100 mg	−25.100	1.66
10	DNA of Meatball D 50 mg	7388.1	1.01
11	DNA of Meatball D 100 mg	−28.050	1.54

Table 2. Data of Nanophotometer Results of DNA Dilution

No	**Sample**	**DNA Concetration (ng/μL)**	**Purity A_{260}/A_{280}**
1	DNA of Beef	266.15	1.80
2	DNA of Meatball A 50 mg	108.35	1.75
3	DNA of Meatball B 50 mg	281.00	1.84
4	DNA of Meatball C 50 mg	772.75	2.10
5	DNA of Meatball D 50 mg	636.35	2.04

For DNA amplification, 50 mg samples were used because the porcine DNA samples were of good purity, while the A, B, C, and D DNA samples were used for DNA amplification because the sample concentrations were quite high. Samples with a high enough concentration of 10x dilution are then carried out by mixing 5 μL DNA and 45 μL Nuclease-free water. Nanophotometer Results of DNA Dilution are presented in Table 2.

After the 50 mg DNA was diluted, it was seen that the DNA purity was much better than before the dilution. The purity value of DNA extracts of more than 2.0 indicates that the DNA extract still contains contaminants from protein compounds. If the purity value of the DNA extract is less than 1.8, it indicates that the DNA extract still contains phenol residues and other solvent contaminants. The phenol-chloroform extraction method with a sample weight of 50 mg (Table 2) was able to produce the concentration and purity of DNA extract required for molecular analysis [16].

Description:
A: Meatball sample A 50 mg

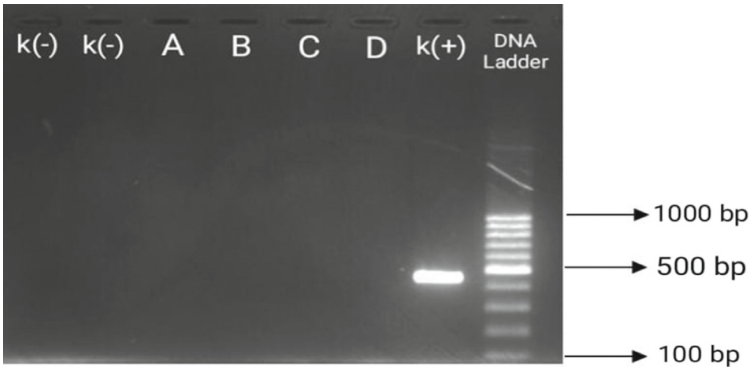

Fig. 1. Pork DNA amplification and meatball samples

K(−): Negative control NFW
B: Meatball sample B 50 mg
K(−): Negative control of beef DNA
C: Meatball sample C 50 mg
K(+): Positive control of pork DNA
D: Meatball sample D 50 mg

In the results of DNA amplification (Fig. 1) with Sus-F and Sus-R primers, based on the electropherogram obtained, only positive control bands (pork DNA) were seen, while meatball DNA samples A, B, C, and D did not show any DNA bands. The first negative control (nuclease-free water was added instead of template DNA) was used as a control in PCR, to determine whether there was cross-contamination between samples. From the electropherogram on the negative control, there was also no DNA band. The second negative control (bovine DNA) did not show this band because the primer used was a pork-specific primer that only amplifies pork DNA. In the positive control (pork DNA) the resulting band was quite clear, single and thick, and in accordance with the target size of 467 bp. Thus, based on these results, it can be concluded that the meatball sample used did not contain pork.

Based on the results of this study, it can be said that the isolation method with phenol-chloroform and the amplification method with conventional PCR can be used as a halal test for the detection of pork genes in meat-based food samples that have gone through a long processing or cooking process.

4 Conclusion

DNA isolation using the phenol-chloroform method with a sample weight of 50 mg was able to produce good quality and quantity of DNA and the DNA amplification method with conventional PCR was able to detect pork DNA well, the resulting band was thick, single, clear/bright, and in accordance with the size, the target is 467 bp.

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