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Original Research

Optimization of Specific PCR Conditions for Cows (Bos taurus) in Rendang Samples for Molecular-Based Halal Tests.

Nurfadillatun Nisa Wijaya[1], Afifatul Achyar[1]*, Dwi Hilda Putri[1], Siska Alicia Farma[1]
[1] Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang, Jl. Prof. Dr. Hamka, Air Tawar, Padang, Sumatera Barat
*corresponding author
E-mail address: afifatul.achyar@gmail.com

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Abstract

Rendang is one of the popular foods in Indonesia made from beef as the main ingredient, which has great potential in tourism development. As a country that has a Muslim majority population, this is very influential for the halal tourism industry. Halal tourism is the parent of tourism in accordance with Islamic principles. The issue of halal food is a sensitive issue for the community. The rise of mixing of non-halal ingredients such as pork in processed food products has worried the public, especially for adherents of the Islamic religion. However, this can be detected molecularly using the Polymerase Chain Reaction (PCR) technique which will amplify the DNA of the target gene of the species to be identified. Each gene has a primer with a different concentration and annealing temperature, so that prior to PCR with research samples, it is necessary to optimize the primer concentration and annealing temperature in order to obtain the appropriate PCR composition and conditions so as to obtain optimal PCR results. In this study, optimization of bovine-specific BOS primers that amplify the ND5 gene in mitochondrial DNA was carried out, namely ND5 (NADH dehydrogenase subunit 5). The results showed that the optimum annealing temperature was 48.2ºC, the optimum primary concentration was 0.5 µM.

Introduction

Rendang is one of the popular foods in Indonesia (Mawardi 2021). Not only in Indonesia, rendang was named the best food in the world by CNN Go's version in 2011. In 2017, rendang was again ranked first in the category of World's Best 50 Food according to CNN Travel.

Because of the popularity of rendang, Indonesia has great potential in tourism development. As a country that has a Muslim majority population, this is very influential for the halal tourism industry. Halal tourism is the parent of tourism in accordance with Islamic principles (Prananta et al. 2018). The issue of halal food is a sensitive issue for the community (Ali 2016). The widespread mixing of non-halal ingredients such as pork in processed food products such as beef meatballs has worried the public a lot, especially for adherents of the Islamic religion (Wahyuni et al. 2019).

However, this can be detected molecularly using the Polymerase Chain Reaction (PCR) technique which will amplify the DNA of the target gene of the species to be identified. The PCR technique has a sensitive ability to detect the presence of pork in fresh meat or processed products mixed with other ingredients (Fibriana et al. 2012). The same thing was also stated by Triasih (2020) that the analytical

method used in detecting the basic ingredients used in a food product is by using protein and DNA-based analysis methods, including PCR amplification of mitochondrial DNA, PCR-RFLP analysis, and PCR Sequencing.

The PCR technique has a sensitive ability to detect the presence of pork in fresh meat or processed products mixed with other ingredients (Fibriana et al. 2012). PCR (Polymerase Chain Reaction) is a revolutionary method developed by Kary Mullis in the 1980s. PCR is based on using the ability of DNA polymerase to synthesize new strands of DNA that complement the offered template strands. Since DNA polymerase can add nucleotides only to a pre-existing 3'-OH group, it requires a primer that can add the first nucleotide (ncbi.nlm.nih.gov). PCR is the method of choice for DNA amplification, both as an analytical and diagnostic technique, because it rapidly produces a large number of copies of the target DNA sequence (Marmiroli & Elena 2007).

The PCR technique can be used to amplify DNA segments millions of times in just a few hours. Each gene has primers with different annealing concentrations and temperatures, so that prior to PCR with research samples, it is necessary to optimize the primer concentration and annealing temperature in order to obtain the appropriate PCR composition and conditions so as to obtain optimal PCR results (Setyawati and Siti 2021; Yuenleni 2019).

In the PCR process, the primer functions as a barrier to the target DNA fragment to be amplified and at the same time provides a hydroxy group (-OH) at the 3' end which is needed for the DNA extension process (Handoyo & Ari 2000). It is generally accepted that the optimal length of PCR primers is 18-22 bases. This length is long enough for adequate specificity, and short enough for the primer to bind easily to the DNA template temperature annealing (Borah 2011).

This variation in annealing temperature is obtained by calculating the average melting temperature (Tm) of the Forward and Reverse primers and then subtracting 5. Because the annealing temperature is usually 5°C below the actual primary Tm (Yuenleni 2019). melting temperature is an estimator of the stability of DNA hybrids and is important in determining the annealing temperature. Too high Tm results in insufficient primer-template hybridization resulting in low PCR product. Too low Tm can lead to non-specific products caused by many base pair mismatches. Incompatibility tolerance was found to have the strongest influence on PCR specificity (Borah 2011).

The important steps in the PCR process include denaturation, annealing and extension. Annealing is the step where the primer attaches to the DNA template. Primers can stick to the DNA template if the temperature used is the optimum temperature, so the temperature used in the annealing stage is an important factor in the success of DNA amplification by the PCR method. Annealing temperature that is too high can lead to unsuccessful DNA amplification, while annealing temperature that is too low can cause primers to attach to an unspecified site (Amanda 2019).

Materials and Methods

2.1. Materials

The tools used for DNA amplification are micropipette size 0.5-10 µL and size 10-100 µL, PCR tube, microtube 1.5 mL thermal cycler (PCR machine), vortex, spin downrack microtube PCR cooling blocks, ice box. The equipment used for electrophoresis is microwave, electrophoresis apparatus, micropipette, well comb, and PCR cooling blocks. And for the analysis of electrophoresis results using a UV transilluminator.

The materials used for DNA amplification were 2x MyTaq HS Red Mix Bioline PCR Master Mixprimer forward (Bos-F) and reverse (Bos-R), tips, nuclease-free water (NFW) and DNA samples (the results of the isolation of wet rendang and beef). The materials used for electrophoresis were agarose, TAE buffer (Tris-acetate-EDTA), GelRed, ladder 100 bp loading dye and PCR samples.

2.2. Methods

To obtain the right annealing temperature, PCR optimization was carried out using Gradient PCR (Margarwati & Muhamad Ridwan 2010). First, a gradient PCR reaction composition was mixed with a total volume per tube 10 µL consisting of 2x MyTaq HS Red Mix Bioline PCR Master Mix with a final concentration of 1X as much as 5 µL. 10 M Primer Bos-F and 10 M Primer Bos-R with the final concentration of each primer being 0,4 µM as much as 0,4 µL. 1 µL of bovine DNA sample (positive control) with a final concentration <250ng. NFW (Nuclease-Free Water, 3.2 µL ofThen set the temperature conditions and the PCR cycle (PCR program) on the PCR machine. In the initial denaturation stage, set at a temperature of 95 ºC for 3 minutes, the next step is to repeat the cycle 35 times which consists of a further denaturation stage at a temperature of 95ºC for 15 seconds, then an annealing step at a gradient temperature (Tm primary ± 5 ºC) for 15 seconds, and the Elongation stage at 72ºC for 10 seconds. Then in the last stage, namely the final elongation at a temperature of 72ºC for 5 minutes and only one repetition of the cycle. Furthermore, the results were analyzed by electrophoresis, which is the optimum annealing temperature based on the thickest DNA band, single strand and the appropriate amplicon size.

The optimization of the BOS primer concentration was varied from the range of 0.2-0.6 µM. The composition of the PCR reaction in the optimization of the BOS primer concentration is with a total volume per tube of 10 µL consisting of 2x MyTaq HS Red Mix Bioline PCR Master Mix with a final concentration of 1X as much as 5 µL, 1 µL of bovine DNA sample (positive control) with a final concentration of <250ng, and Primer Bos-F and Primer Bos-R with varying concentrations of each primer 0.2-0.6 µM as much as 0.2-0.6 µL. Next, NFW (Nuclease-Free Water) was added to make up the volume to 10 µL. Then set the temperature conditions and the PCR cycle (PCR program) on the PCR machine. In the initial denaturation stage, set at a temperature of 95ºC for 3 minutes, the next step is to repeat the cycle 35 times which consists of a further denaturation stage at a temperature of 95ºC for 15 seconds, then an annealing step at a gradient temperature (Tm primary ± 5ºC) for 15 seconds, and the Elongation stage at 68ºC for 30 seconds. Then in the last stage, namely the final elongation at a temperature of 68ºC for 5 minutes and only one repetition of the cycle. Furthermore, the results were analyzed by electrophoresis, which is the optimum annealing temperature based on the thickest DNA band, single strand and the appropriate amplicon size.

The PCR sensitivity test was carried out with several levels of template using serial dilutions. Serial dilutions were carried out by adding 5 µL of NFW (Nuclease-Free Water) and 5 µL of DNA samples. Then set the temperature conditions and the PCR cycle (PCR program) on the PCR machine. In the initial denaturation stage, set at a temperature of 95oC for 3 minutes, the next step is to repeat the cycle 35 times which consists of a further denaturation stage at a temperature of 95oC for 15 seconds, then an annealing step at a gradient temperature (Tm primary ± 5ºC) for 15 seconds, and the Elongation stage at 68 oC for 30 seconds. Then in the last stage, namely the final elongation at a temperature of 68ºC for 5 minutes and only one repetition of the cycle. Furthermore, the results were analyzed by electrophoresis.

The PCR results were visualized by electrophoresis of 1.5% agarose gel that had been prepared previously. The 1.5% agarose gel is cooled briefly at room temperature so that it is not too hot, then poured into a gel mold and fitted with a well-forming comb, then allowed to stand until the agarose gel hardens. Next, the well-forming comb is removed from the agarose gel, then the agarose gel is placed with the mold in the electrophoresis chamber. Then fill the electrophoresis chamber with TAE1X buffer until the agarose gel is submerged. Then the agarose gel well was filled with a mixture of samples and a mixture of ladder DNA. In the sample mixture, 5 µL of parafilm/tape was added to Gel Red 1:1000 and 5 µL of DNA/PCR product samples, then homogenized using a micropipette. In the DNA ladder mixture, 15 µL of Gel Red 1:1000 was added on top of parafilm/tape, 1 µL of loading

dye, and 3 µL of 100 bp DNA ladder. Then the electrophoresis process is carried out with a voltage of 100 volts for 30 minutes. After that the results of the electrophoresis were seen using a UV Transilluminator.

Results and Discussion

In this study, cow-specific primers were used, namely BOS-F and BOS F. The order of primers for BOS-F and BOS-R can be seen in table 1.

Tabel. 1 Primer to be used during PCR

PCR	Primer	Sequence	Reference
Cow Specific (206 bp)	Bos-F	5'-ACC CTT GAT TGG ACT AGC ATT-3'	(Chatri *et al*., 2021)
	Bos-R	5'-TAC ATC CAA TCT ATT ACA TTA TGC T-3'	

The results of optimization of annealing temperature can be seen in Figure 1

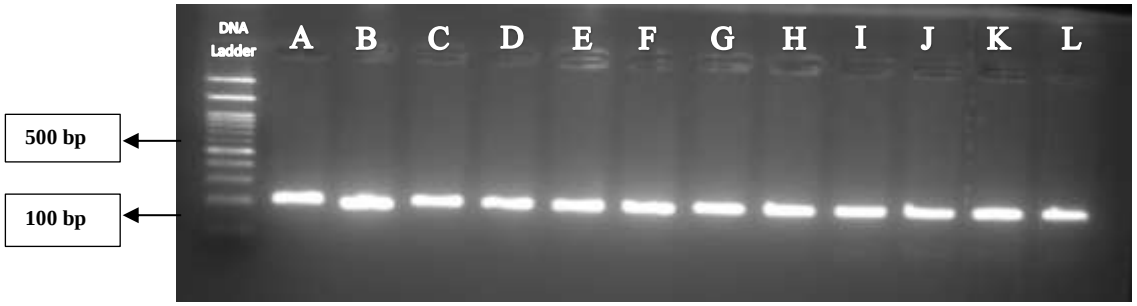

Figure 1. Electrophoresis results of PCR products with annealing temperature using primers BOS-F and BOS-R in cattle.

Description Annealing Temperature :

A	: 47,7 ºC	E	: 51,0 ºC	I	: 55,5 ºC
B	: 48,2 ºC	F	: 52,1 ºC	J	: 56,4 ºC
C	: 49,0 ºC	G	: 53,3 ºC	K	: 57,2 ºC
D	: 49,9 ºC	H	: 54,4 ºC	L	: 57,7 ºC

The results of amplification of the ND5 gene region in cattle obtained DNA bands measuring about 200 bp with an optimum annealing temperature of 48.7 C. In the picture, the resulting band at B is thicker, single, and according to the target size (206 bp). The annealing stage is an important step because it is the stage of primer attachment after the DNA double chain is open. The selection of annealing temperature that is too low will cause non-specific attachment, while a temperature that is too high can make it difficult for the primer and template DNA bonds to form. The annealing temperature used must be adjusted to the Tm or melting temperature of the primer and the length of the primer (Amanda 2019). The Tm temperature of the BOS-F and BOS-R primers were 52.7°C and 53.5°C, the Tm temperature used to determine the annealing temperature was the lowest Tm temperature of 52.7°C and lowered by 5 °C so that used Tm 47.7°C.

In this study, optimization of bovine-specific BOS primers that amplify the ND5 gene in mitochondrial DNA was carried out, namely ND5 (NADH dehydrogenase subunit 5). Genes in mitochondria have more copies, making them easier to detect than chromosomal DNA. This higher number of DNA copies is very important because the heating process when cooking food indirectly degrades the DNA present in the raw material of the food.

Optimization of primer concentration aims to find the optimum primer concentration conditions to obtain PCR conditions with high efficiency. In this study, the variation of the primary concentration was 0.2 M; 0.3 M; 0.4 M; 0.5 M; and 0.6 M.

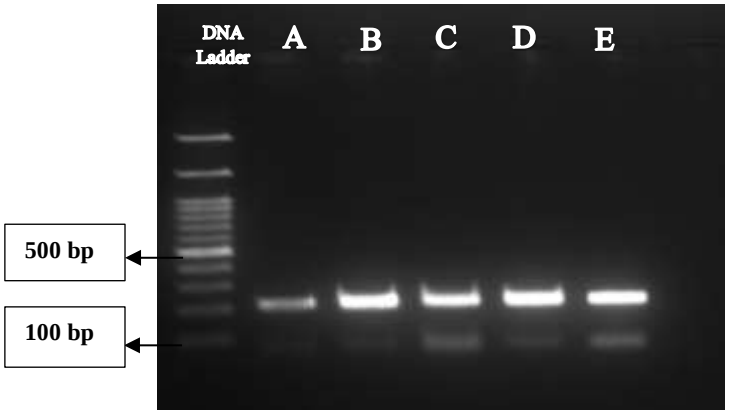

Figure 2. Electrophoresis results of PCR products with variations in primer concentrations of BOS-F and BOS-R.

Description :

A	: 0,2 µM	D	: 0,5 µM
B	: 0,3 µM	E	: 0,6 µM
C	: 0,4 µM		

The figure, it can be seen that the resulting band on D is thicker, single, and according to the target size (206 bp). So for the next PCR reaction using the optimum primer concentration of 0.5 µM. Sensitivity is carried out to determine the minimum concentration of DNA that can be detected by the PCR method used in the test laboratory.

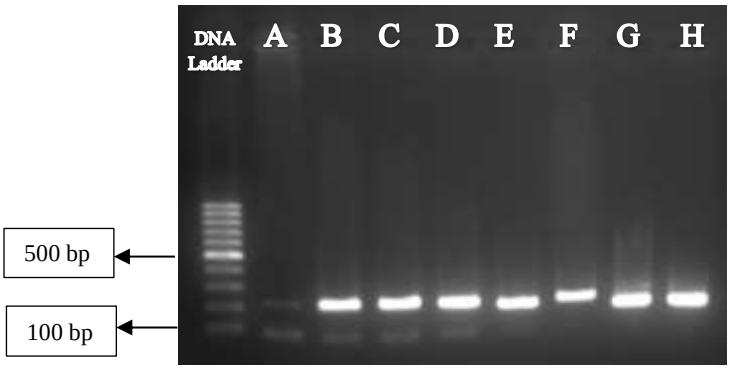

Figure 3. Electrophoresis results of PCR products with variations in the concentration of wet rendang template DNA

Description :

A	: NFW (kontrol negative)	E	: 12,5 ng/µL
B	: 1,5625 ng/µL	F	: 25 ng/µL
C	: 3,125 ng/µL	G	: 50 ng/µL
D	: 6,25 ng/µL	H	: 100 ng/µL

In the sensitivity test using a negative control as a comparison, if no DNA bands are observed, the test results do not experience cross-contamination. The variation of the template DNA concentration was carried out by serial dilutions by adding 5 µL of NFW (Nuclease-Free Water) and 5 µL of DNA samples. Figure 3. shows that at a concentration of 100 ng/µL to a concentration of 1.5625 ng/µL the DNA band still looks thick and clear. As shown in the figure, NFW as a negative control was cross-contaminated.

Conclusions

Based on the results of the study, it was found that the optimal annealing temperature for BOS primers was 48.2 and the optimal concentrations of BOS-F and BOS-R primers were 0.5

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