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Real-Time PCR-based detection of bovine DNA by specific targeting on cytochrome-B

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ABSTRACT

The design of specific primers is an interesting research topic such that it offers selective, specific, and effective DNA analysis using real-time PCR. This research was intended to detect bovine DNA using real-time PCR and specific primers to ensure the halal authenticity of food products**.** Primers of bovine DNA sequences were designed in the NCBI and Primer-BLAST programs. The outcome validation was assessed using several parameters, namely specificity, repeatability, and linearity by real-time PCR. Primer specificity test was performed on fresh tissue (pork and negative control), while the repeatability test used six replications and was based on the calculated coefficient of variation (CV). In the linearity test, six different DNA concentrations (50000, 10000, 5000, 500, 100, and 50 pg/µL) were examined to obtain the efficiency value. Using the specific primer from Cytochrome-B, the real-time PCR could specifically identify the presence of bovine DNA at the optimum annealing temperature of 58.7^0C. The repeatability analysis yielded a coefficient of variation (CV) of 0.57 %, while the linearity test produced an efficiency value of 206 %. These figures confirm that the method employed in this study is not only specific but also sensitive and reliable for detecting bovine DNA. Real-time PCR using specific primer targeting on the cytochrome-B region of bovine DNA (forward: CTACTGACACTCACATGAATTGG; reverse CACTAGGATGAGGAGAAAGTATAGG) can be used to identify bovine DNA and distinguish it from porcine DNA.

Keywords: primer, Cytochrome-B, bovine DNA, specific, real-time PCR

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INTRODUCTION

For Muslims, food products are not safe without halal status. It follows a belief that halal is the highest quality of food products and that it already includes food safety. Researchers continuously seek or develop analytical methods that can detect and even quantify the presence of non-halal components in food products. More importantly, this attempt answers the necessity of feeling assured that foodstuffs are derived from halal materials, one of which is raw products from cattle.

The identification of meat in food products has lately adopted DNA-based analytical methods. Aside from their fast and specific procedure, these methods rely on DNA that is advantageously more stable even though it has been exposed to physical and chemical processing (Iwobi *et al.*, 2012). Accordingly, this research seeks a valid and reliable analytical method to detect the presence of beef gelatin through specific primers from edible composition to ensure the halal authenticity of pharmaceutical products.

Compared to a conventional polymerase chain reaction (PCR), real-time PCR provides a more sensitive, simpler, and faster analysis. It is the development of the PCR method that allows the results of amplification to be directly observed and analyzed quantitatively using DNA dyes and fluorescent trackers (Sudjadi, 2008). Another use of real-time PCR is for the detection of single nucleotide polymorphisms and accurate determination of melting temperature from the resultant real-time PCR curves. Its development extends to molecular biology, especially for halal analysis. An example includes the detection of pork in consumables (Himawati, 2013; Martin *et al.*, 2009; Soares *et al.*, 2013; Taufiq, 2014). In general, real-time PCR analysis is divided into three parts, namely the exponential phase, the linear phase, and the flat phase. In the exponential phase, it is possible to obtain PCR amplification products with efficiencies close to 100%, which means DNA duplication occurs in this phase. The percentage of efficiency depends on PCR conditions, the characteristics of the designed primers, the purity of the DNA print, and the length of the amplification product. PCR results can be analyzed by correlating Cq (Ct) values with DNA concentration. A better correlation is obtained by relating Cq number with the logarithm-transformed concentration because it yields a linear and significant correlation for each concentration. PCR data quality is said to be good if the PCR amplification efficiency is close to 100% (Yuan *et al.*, 2006).

At this time, many programs facilitate the designing of primers. Users can conveniently design primers by modifying the length of the primer, the length of the DNA amplification product, and the guanine-cytosine (GC) content according to the research needs. Moreover, these programs are integrated with the DNA sequence database, meaning that after the primer is designed, the specificity of the target DNA and any mismatches that may arise can be observed directly. Besides, they enable the estimation of whether the designed primers are attached to the DNA sequences of other species or specific to the target DNA. Primer specificity is essentially considered in the PCR technique; in other terms, specific primer design is crucial to the success of a PCR-based analytical method (Sudjadi, 2008).

One of the goals of this research is to obtain a specific primer from bovine mitochondrial DNA for DNA identification in real-time PCR. Bovine DNA in this research was obtained from *Bos taurus*, a species of cows originating in Europe. This group eventually spread to various parts of the world, including Indonesia. Since the raw material of gelatine mostly comes from *Bos taurus*, this research uses and focuses on this cow species.

MATERIALS AND METHOD

Materials

The primer was designed with software hosted by the NCBI website and subsequently ordered from PT Genetika (Jakarta, Indonesia). Beef (*Bos taurus*) and pork (*Sus scrofa*) meat were purchased from the traditional markets in Yogyakarta. The species-specific primers were

designed and tested with this software. The other solvents and chemical reagents were bought from E. Merck (Darmstadt, Germany).

The research instrument included real-time PCR CFX 96 (Biorad, USA), and the procedure was processed at LPPT-UGM.

The designing phase of DNA specific primer

The bovine DNA primer was designed using NCBI-Primer BLAST software by inputting the desired criteria data. The resultant primers were then confirmed for their specificity for *Bos taurus*.

DNA isolation and subsequent purity test

DNA isolation was carried out by grinding 200 mg of each meat sample with a mortar and then inserting it into a 2ml microtube. A warm lysis buffer was added as much as 700 µL to the microtube, and 10 µL proteinase-K was added later. The mixture was homogenized with a vortex device, incubated in a water bath at 65°C for 2 hours, and then vortexed until the meat sample was destroyed. Phenol-KIAA solution with 1: 1 volume was added to the microtube. Afterward, the mixture Submitted: with a shaker for 30 minutes. The samples were centrifuged at a speed of 12,000 rpm for 5 minutes, producing two layers in each sample. The supernatant, the liquid at the top payer, was collected, placed in a new microtube, and then added with 2-propanol with a volume of 1: 1. The mixture was incubated in the freezer for 15 hours then centrifuged again at a speed of 12,000 rpm for 5 minutes. Afterward, the DNA precipitated and was seen at the bottom of the microtube. The supernatant from centrifugation results was then discarded. A total of 500 µl of 70% ethanol solution was added into the microtube and centrifuged at 12,000 rpm for 5 minutes. After centrifugation, the supernatant was completely removed and allowed to dry, so that DNA pellets could form at the bottom of the tube. The DNA pellets were dissolved in 100 µL TE buffer, homogenized, and incubated at 50°C. The isolated sample was then stored in the freezer (Olson, N. D., and Morrow, J. B., 2012).

The results of DNA isolation were analyzed for its purity by measuring the absorbance of 20 µL DNA solution in 980 µL sterile aqua at the wavelengths (λ) 260 nm and 280 nm. Meanwhile, the DNA concentration was calculated from the absorbance value at λ 260 nm and multiplied by the dilution factor and the absorption constant (50 µg / mL).

Real-Time PCR Analysis

The real-time PCR analysis was performed using a reaction mixture (20 µL) consisting of 10 µL SYBR Green® universal PCR master mix, 1 µL forward and reverse primer, 1 µL DNA template (50 ng), and 7 µL free nuclease water. The temperature was set at 95°C for 30 seconds, followed by 30 cycles at a temperature of 95°C for 5 seconds for the denaturation stage. The next step was the primer attachment. This procedure was performed at the optimized primer attachment temperature for 10 seconds and 72° C for another 10 seconds to create the advanced stage (extension). The melting curve was carried out at the temperature of 60-90°C with a slope of 0.5°C/2 seconds (Himawati, 2013).

Optimization of annealing temperature

The annealing temperature primer that had been designed for rat DNA was optimized at the temperature range of 52 to 62°C using real-time PCR (Bio-Rad, 2006).

The specificity of primer

The primer specificity was confirmed by amplifying 50 ng/µL DNA of beef, pig, and negative control (without DNA) called NTC (No Template Control).

Repeatability test

The repeatability of the method was assessed based on the coefficient of variation (CV). This value was obtained from 6-time replication of the amplification of isolated DNA from fresh beef at the concentration of 50 ng/µL.

Data Analysis

The real-time PCR results were analyzed using a standard curve created by comparing Cq values (Quantification circle) with the log-transformed DNA concentration. The real-time PCR efficiency (E) was calculated using the formula: E = (10 (-1 / slope) - 1) x 100 (Bio-Rad, 2006).

RESULTS AND DISCUSSION

Designing primer is the initial stage of real-time PCR analysis. In this study, the primer design aimed to obtain a specific primer arrangement of bovine mitochondrial DNA (*Bos taurus*). Sudjadi (2008) states that the determining factor of the success of PCR is the use of primers that are specific to the target or, in other words, specific primers are vital to achieving an optimal result. Mitochondrial DNA can be targeted for genes or molds in the PCR process. It has many advantages, including the incorporation of mitochondrial DNA—which is inherently more stable under various conditions during the production process. A cell can contain thousands of copies of mitochondrial DNA, while the number of samples required in PCR analysis is small. With these advantages, PCR analysis using mitochondrial DNA as a target is, therefore, a relatively cheaper, more straightforward, and more sensitive method (Nuraini *et al.*, 2012).

Mitochondrial DNA as a target has been widely used in research employing both conventional PCR and real-time PCR. Aida (2005), Rahman (2014), and Camma (2012) have developed PCR methods with mitochondrial DNA as a target sequence. Mohamad *et al.* (2013) suggest that the template used in real time-PCR, either mitochondrial DNA or nuclear DNA, significantly affects the sensitivity and specificity of the analysis method.

The PCR amplification results were detected with SYBR Green. SYBR Green is a fluorescence-binding agent with double-stranded DNA that can be detected at λexitation= 498 nm and λemission= 522 nm, and the intensity of fluorescence that arises is read by the detector tool. Compared to other detectors, SYBR Green can be universally used in various prints because of its non-specific nature that it can bind to any double-stranded DNA, and it is cheaper, simpler to use, and more stable at elevated temperatures. Also, it does not interfere with DNA Polymerase (McPherson and Simon, 2006).

The primer was designed using the Primer-BLAST software available on the NCBI website. The organism of target used in the primer design for this study was *Bos taurus*, in particular, the cytochrome b gene region with the GenBank code AF492351. The primer candidates were obtained from the primer designs produced by BLAST software for other organisms. The in silico preliminary testing of the primer design yielded a pair of primers that were later used in this study, as shown in Table I.

Table I. The primer design of the Cytochrome-B region of *Bos taurus*

Primer		Length	Tm	%GC	Product Length
Forward	CTACTGACACTCACATGAATTGG	23	57.21	43.48	99 bp
Reverse	CACTAGGATGAGGAGAAAGTATAGG	25	57.44	44.00	

Table II shows the results of the quantitative analysis, namely the A260/A280 ratio, of DNA isolates using a spectrophotometer. Clark (2005) affirms that pure DNA has a ratio of 1.8. Accordingly, ratio<1.8 indicates protein contamination, while ratio>1.8 represents RNA contamination. The quantitative analysis revealed that the isolation included imperfect washing,

meaning that the DNA obtained was not pure. Codex Alimentarius Commission (2010) explains that high DNA purity allows optimal amplification that affects the validity of a PCR method.

Table II. The results of quantitative analysis and purity test of DNA meat isolates by spectrophotometry

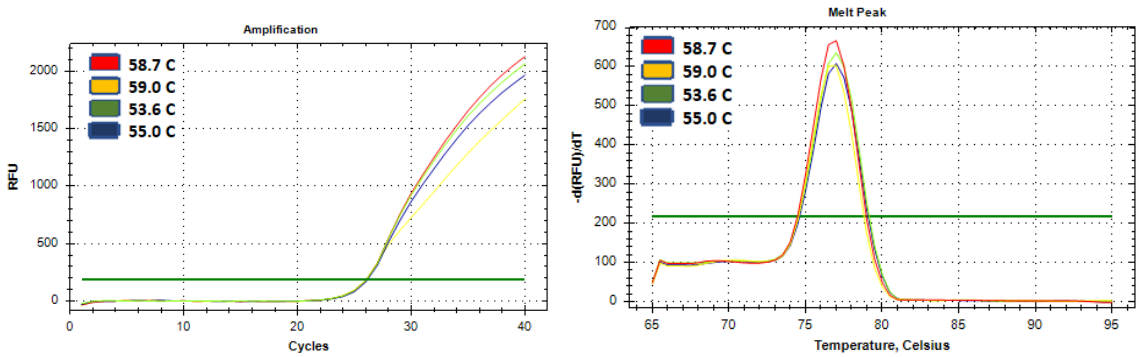
Types of Meat	Concentrations (µg/mL)	A_{260}/A_{280} Ratio
Porcine	1952.68	2.00
Bovine	1897.80	1.50

A primer is designed with a theoretical melting temperature (Tm) based on the GC content of the primer. Muladno (2010) states that the primer attachment temperature is usually in the range of 50-60°C or approximately 5°C below the Tm of the design primers. However, temperature optimization is a necessary element to achieve optimal PCR results that are specific to bovine DNA. In this study, the temperature was optimized using the bovine and porcine DNA targets from a range of primer attachment temperature, which was 53.6 - 59.0°C, based on the estimated Tm of the designed primers (Table III).

Table III. The optimized annealing temperatures of primers from bovine DNA

Optimized Temperature	Cq	Melt Temperature	Peak Height
58.7	26.03	77.00	666.82
59.0	26.01	77.00	603.13
53.6	26.03	77.00	635.55
55.0	26.11	77.00	608.09

The results of the optimization of annealing temperature are presented in Figure 1. Based on the figures, the exact annealing temperature used in the PCR real-time test protocol is 58.7°C. At this temperature, the maximum amplification results and the highest melting temperature among other optimization temperatures are achieved.

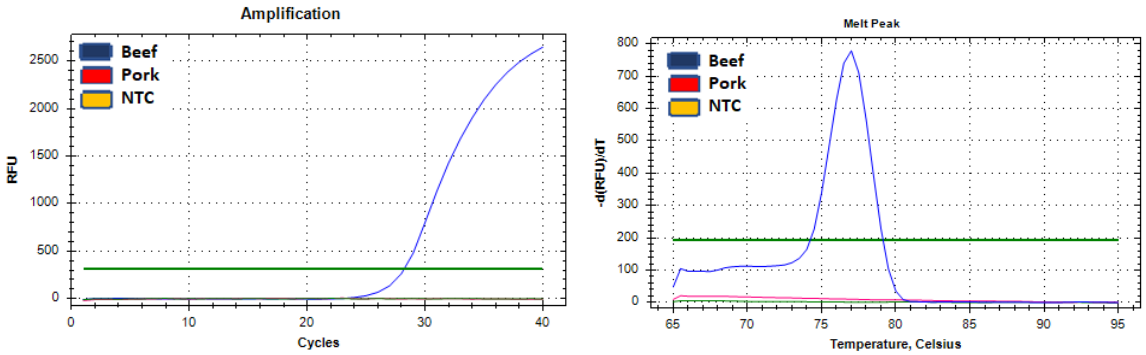


(A) **(B)**

Figure 1. The results of the amplification of bovine DNA and the optimization of annealing temperature of the designed primers (A) Cycles (B) Melting temperature

PCR strongly depends on the specificity of primer because a specific primer design can lead to the success of any PCR-based analytical methods (Sudjadi, 2008). Specific primers are primers that can be used to amplify the target DNA, rather than amplifying the DNA of other species as universal primers do. In this study, the specificity testing was carried out only on the porcine DNA. This method is a necessary step to initiate the test on samples of gelatin because the available gelatin on the market mostly comes from pigs and cows. The negative control, NTC (no DNA content), was also subjected to a specificity test. Based on the results of the specificity test (Figure 2), it appears that only bovine

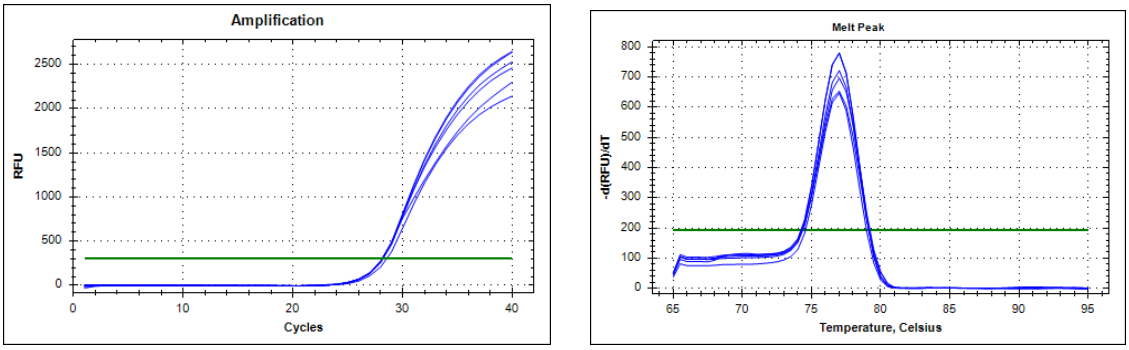
DNA is amplified, whereas NTC and porcine DNA are not. This result indicates that the primer used in this study is specific.



(A) **(B)**

Figure 2. The results of the amplification (A) of the designed primers, with Cq: 28.22 and (B) Tm: 77.00

The test of method repeatability was carried out by observing the amplification results of the amplification of rat meatball samples with a concentration of 100% and the amplification products of rat meat that was replicated six times to see the consistency of the real-time PCR results (Figure 3 and Table IV). The Relative Standard Deviation (RSD) or CV of Cq is the basis for making decisions of whether or not the method is consistent. Codex Alimentarius Commission (2010) sets the acceptable coefficient of variation (CV) for a repeatable PCR analytical method at 25%. The CV of Cq from the amplification of bovine DNA with a concentration of 50 ng/µL was 1.91%, while the CV of Cq from rat meat amplification was 1.01%. These results indicate that the method meets the repeatability requirements.



(A) **(B)**

Figure 3. The repeatability test results of DNA amplification from beef isolates with the DNA concentration of 50 ng/µL (A) Cycles (B) Melting Curve

Table IV. The repeatability of PCR analytical method for bovine DNA

Sample	Cq	Tm	Peak Height	average Cq	SD	CV (%)
Bovine DNA concentration 50 (ng/µL)	28.59	77.00	647.04	28.27	0.1621	0.57
	28.22	77.00	778.70			
	28.19	77.00	780.05			
	28.26	77.00	721.29			
	28.17	77.00	696.51			
	28.17	77.00	653.12			

Linear regression equations were also obtained from the standard curve of amplification reaction to DNA isolates from beef (Figures 4 and 5), with R2 values=0.966, slope= -2.059, and y-intercept= 28.830, meaning that the curves meet the linearity criteria.

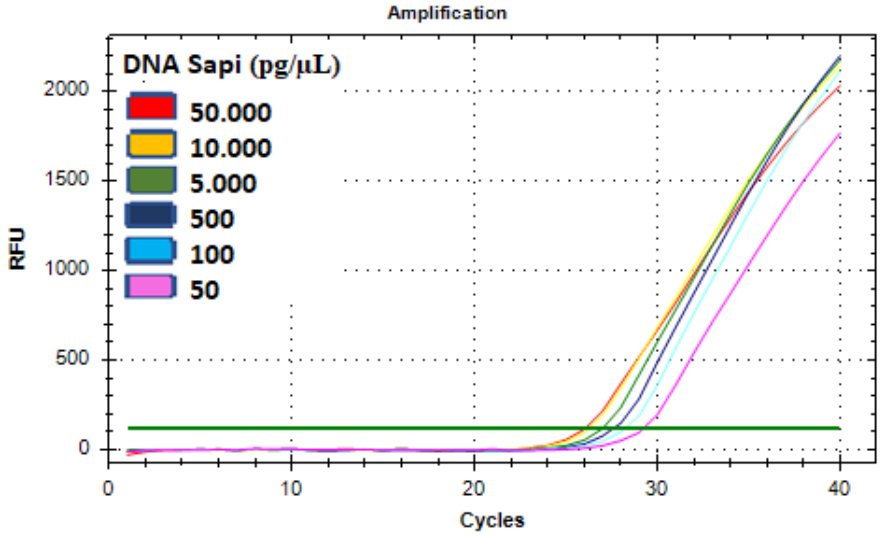


Figure 4. The results of bovine DNA amplification at various concentrations

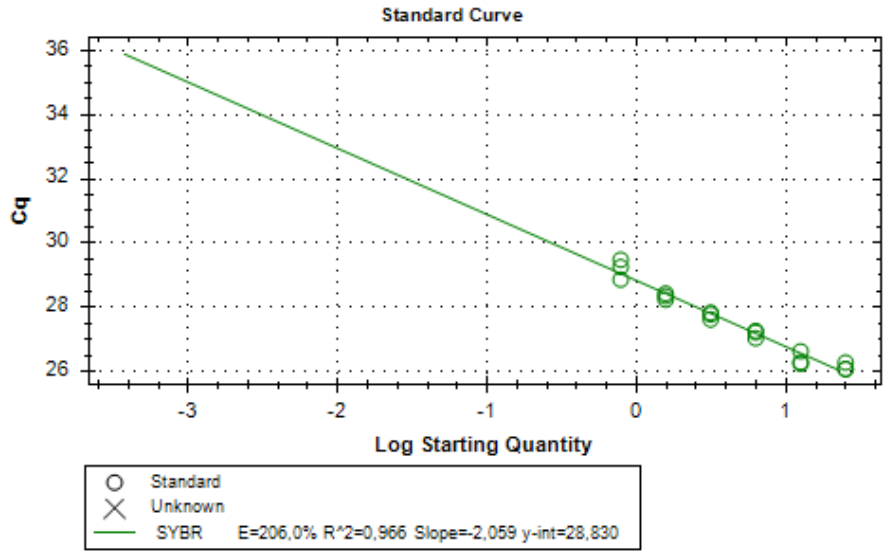


Figure 5. The standard curves of bovine DNA amplification at various concentrations

The standard curve between log-transformed bovine DNA concentration and Cq (Figure 5) shows an efficiency (E) of 206.0%. This percentage indicates imperfect results due to high-efficiency values. Bio-Rad (2006) states that the results of PCR amplification are considered as good or acceptable if the efficiency value is close to 100% (90-105%) and the value of R2 is >0.980. If amplification efficiency values are higher than 100%, the thermocycling conditions and the reactions occurring in PCR components are less than optimal (Life Technologies, 2012). Efficiency values can be influenced by several factors, namely the presence of inhibitors in the master mix PCR, the purity of the reagents used, inconsistencies in the small volume piping process—which results in low accuracy, and the testing ability that depends on the primer specificity and target sequence length of the printed DNA (Svec *et al.*, 2015; Muhammed *et al.*, 2015). Extremely high efficiency indicates that the target DNA duplicated in each cycle is disturbed by the presence of an inhibitor during the reaction, whereas too small efficiency values represent imperfect primer attachment. Both cases result in less exponential template amplification in each cycle.

CONCLUSION

Specific primers 5 '- CTACTGACACACACGAGAATTGG - 3' (forward) and 5 '- CACTAGGATGAGGAGAAAGTATAGG - 3' (reverse) from the cytochrome-b region of bovine mitochondrial DNA (*Bos taurus*) can be used specifically to distinguish bovine DNA from porcine DNA (*Sus scrofa*) using real-time PCR method with an optimum temperature of 58.7°C for primers attachment. Therefore, these primers can be used for analyzing the halal authentication of gelatin.

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