

Porcine-specific Primer based on Cytochrome B by Real-Time Polymerase Chain Reaction Method for Identification in Raw Meat

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Abstract—Pork is a type of meat that is often used for counterfeiting products with a composition of beef. This counterfeiting can provide large profits to producers, given the price of pork is far below the price of beef. So we need specific methods to ensure the halal product. The purpose of this study was to obtain a method for identification of pork using real-time PCR instruments. The validation parameters of the PCR real-time method include sensitivity test, linearity test, determination of detection limit and repeatability test. Specific pig primers designed with the NCBI and Primer-BLAST software (5' -CGGAACAGACCTCGTAGAATG - 3' (forward) and 5' -GGTAATGATGAATGGCAGGATAAAG - 3' (reverse) can amplify pig mitochondrial DNA Cytochrome with annealing temperature of 53.20C. Primary specificity is shown by Melting Curve Analysis (MCA) characterized by the appearance of a peak at the melting peak. Specificity testing was done on 4 DNA isolates of fresh meat (pork, chicken, beef, dog) and negative control. The results of the sensitivity test on fresh meat produced an efficiency value (E) of 417.4% and an R-value of 0.908. In the repeatability test, the Coefficient Variation (CV) value of fresh dog meat DNA isolates concentration of 50 ng / μ L was 0.57%.

Keywords—real-time polymerase chain reaction, cytochrome-b, pork (*sus scrofa*), halal authentication

I. INTRODUCTION

The composition of the mixture in the product must be ensured its identity comes from sources that can be accepted by consumers even though it is difficult to observe. Identity of ingredients in the mixture is not easy. It needs analytical methods to identify animal species in food products to protect consumers from counterfeit products. Several analytical methods that can be used for identification are obtained from research results such as Fourier transform infrared (FTIR) spectroscopy, different scanning calorimetry, electronic scent and chromatography [1]. The profile of fatty acids in lard and other animal fats, namely cows, chickens, and sheep have been successfully analyzed by gas chromatography using a flame ionization detector [2]. Analysis of lard in milk fat using

gas chromatography [3] are some examples related to the analysis of non-halal components in food products. [4] Pork, chicken and beef have also been analyzed by determining alcohol content by gas chromatography. [5] The use of two-dimensional gas chromatography combined with Time of Flight Mass Spectrometry (TOF-MS) to distinguish fatty acids in lard, animal fat, and cod liver oil.

Some research on the analysis of non-halal components has also been carried out by several researchers. Identification of non-halal components in various products has been widely carried out, both by physical-chemical methods and by molecular biological methods. Analysis of lard and beef fat has been carried out using pancreatic lipase six enzymes to produce 2-monoglycerides [6]. Identification of pork in meat products is made by real-time polymerase chain reaction [7]. The multiplex PCR real-time method can detect pork, dogs, cats, monkeys, and mice in commercial meatballs [8].

Real-time PCR is one of the development methods of PCR that uses specific primers. The RT-PCR method is capable of producing amplification that can be directly observed and analyzed quantitatively using fluorescent compounds [9]. These compounds correlate to the accumulation of PCR products and are analyzed by Melting Curve Analysis (MCA). The most important thing to consider in the PCR technique is the primary specificity when doing a primary design to determine the success of the PCR method analysis [10]. So that the real-time PCR method that can identify and distinguish sensitive and specific types of meat will be developed in research for analysis of dog meat in market beef meatballs using pig-specific primers.

In meat also contains DNA, the presence of DNA can be analyzed by PCR or Real-time PCR methods [11]. There are three stages in the PCR technique, namely: denaturation, annealing, extension [12]. In the first step, the amplification reaction begins with the denaturation of DNA from a double chain to a single chain using high temperatures (90-97°C). The

next process is primary annealing, which is done by reducing the temperature of the denaturation temperature which then the primer will attach to a specific single DNA chain with the help of enzymes. The final step is the extension of the target DNA sequence by adding a nucleotide using DNA polymerase at 72°C [13].

II. MATERIAL AND METHOD

A. Instrumens and Material

The instrument used in this study include Real-time PCR CFX 96 (Biorad, USA at the UGM Integrated Research and Testing Laboratory (LPPT). Samples used in this study were pork (*Sus scrofa*) obtained in Godean, dog meat (*Canis lupus familiaris*) obtained in Bantul, beef (*Bos taurus*) and chicken (*Gallus gallus*) meat obtained at the Pathuk market, DNA primers that have been designed and tested using software from the NCBI website, ordered from PT Genetika.

B. Method

1. Primary Design

Pig specific DNA primers and probes are designed using IDT DNA online software - "Primer Quest Tools" that fit the criteria. then BLASTED to the DNA of pig chromosomes and some other comparative animals using NCBI's BLASTn (Basic Alignment Search Tool-nucleotide) or BLAST (Basic Local Alignment Search Tool-suite) feature.

2. DNA Isolation

DNA isolation was carried out as [14] with some modifications. Each meat is crushed first using a mortar and stamper, then each weighed about 200 mg of sample, then crushed, added 700 μ L of lysis buffer and 30 μ L proteinase-K into a 1.5 mL microtube, vortex \pm 1 minute. The mixture was incubated in a water heater at 55°C for 2 hours. After incubation, the mixture was vortexed to disintegrate, then a 710 μ L phenol-KIAA solution was added, shaker \pm 30 minutes. Centrifuged at 12,000 rpm for 5 minutes to form 2 layers. The supernatant was transferred into a 2 μ L microtube, then added 2: 1 cold propanol with the sample solution obtained. Incubated in a deep freezer at -8°C for 1 hour. Then centrifuged at a speed of 12,000 rpm for 5 minutes to form 2 layers. The precipitate formed is taken, while the supernatant is removed. 500 μ L absolute ethanol was added, centrifuged at 12,000 rpm for 5 minutes. The supernatant is removed, the precipitate formed is then dried in LAF for 15 hours. After drying 100 ml of TE buffer was added and then incubated at 50°C for 30 minutes. The DNA solution is stored in a freezer at -20°C.

3. Optimization of Annealing Temperature

Optimization of pig's primary annealing temperature has been carried out on the DNA and analysis using thermo fisher software. The protocol for each primer is an initial denaturation temperature of 95°C, 3 minutes, denaturation temperature of 95°C, 15 seconds. Annealing temperature based on primary T_m , 15 seconds and elongation temperature of 72°C, 30 seconds for 30 cycles. Melting Curve Analysis (MCA) starts from 65-95°C with an additional temperature of 0.5°C per second.

4. Test the forward and reverse primary specificity

Primary specificity was confirmed by amplifying 50 ng / μ L pure porcine DNA (*Sus scrofa*) with cattle (*Bos taurus*), chickens (*Gallus gallus*), dogs (*Canis lupus familiaris*), and negative controls (without DNA) called NTC (No Template Control). Each 1 μ L DNA sample (50 ng / μ L was mixed with 10 μ L EvaGreen®, 1 μ L forward primer and 1 μ L reverse primer with a concentration of 1 μ M, and Nuclease-free water to a volume of 20 μ L.

5. Linearity Test

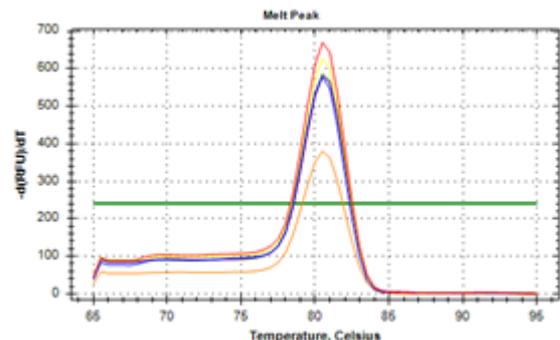
Linearity tests were performed using pure pig DNA isolates in 12 dilution series (5000; 1000; 500; 100; 10 pg / μ L and NTC). The results of the standard curve are made by plotting the Ct value against the log value of DNA concentration. Linear regression is made to calculate slope values, correlation coefficients, and efficiency.

6. Repeatability Test

The repeatability method was assessed based on the coefficient of variation (CV) of 6x replication of the amplification of pure pork DNA isolation results. The CV repeatability testing criteria for PCR are \leq 25% [15].

III. RESULT AND DISCUSSION

The selection of primers is in accordance with the DNA target template sequence (specific), which only binds to the target sequence of the DNA template, and does not form unwanted dimers or amplicons [16]. The specific primer design process consists of two stages, and the first stage is the design of primer pairs that can flank specific regions of the DNA template. The second stage is the primary specificity testing stage for other organisms and similar organisms, and this can be done using the BLAST (Basic Local Alignment Search Tool) software from NCBI. The target organism used in the primary design for this study was *Sus Scrofa* in the cytochrome b gene region with the GenBank code AF034253. Primary candidates obtained from the primary design with the software carried out by BLAST towards other organisms. Based on preliminary testing in silico primary design obtained a pair of primers that will be used in this study, namely Forward: CGGAACAGACCTCGTAGAATG and reverse GGTAATGATGAATGGCAGGATAAAG. This pig primer has melting temperature characteristics of 62°C (F) and 62°C (R), GC contents of 52.4% (F) and 40% (R) with amplicon product of 103 base pair (bp). The validation stages of the primers using the real-time PCR method to be used include specificity, efficiency, and repeatability. Optimization needs to be done to get the annealing temperature which will be used as an analysis protocol in real-time PCR [17].



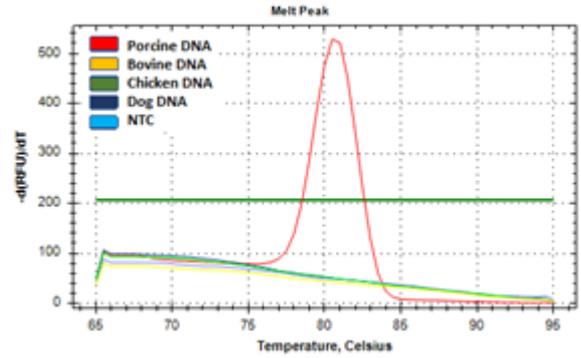
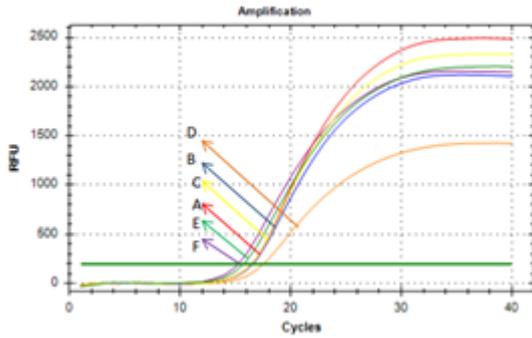


Fig. 1. Optimization results (a) annealing temperatur (ta), and (b) mealing temperature (tm) primer

Fig. 2. Primary pig specificity test results of meat DNA isolates at a temperature of 53.2oC. (a) the resulting amplification curve and (b) the melting peak curve

TABLE I. OPTIMIZATION RESULTS OF ANNEALING DAN MELTING TEMPERATUR

Sample Code	Ta (°C)	Cq	Tm (°C)	Peak height
A	53.2	16.43	80.50	669.78
B	53.0	16.52	80.50	577.90
C	52.7	16.44	80.50	626.65
D	52.1	17.49	80.50	379.98
E	51.4	15.23	80.50	579.85
F	50.8	15.69	80.50	585.97

The criteria for selecting optimum annealing temperature are seen from the high RFU value and the lowest number of amplification cycles [18]. Optimal annealing temperature can cause non-specific amplification results because the annealing temperature is too low or no amplification product is formed in the target area because the temperature is too high. [19] states that the primary annealing temperature ranges from 50o-60oC. Based on Figure 1 and clarified with the table I, annealing temperature of 53.2oC was obtained with a Cq value of 16.43 and Tm 80.50 and the highest peak height of 669.78. Furthermore, the annealing temperature is tested by the primary specification of pigs against other species (beef, chickens, and dogs) and negative control. The annealing temperature is considered optimum if the temperature can only amplify dog DNA without the DNA of other species being amplified.

Based on the specificity test results shown in Figure 2, it was found that by using the primer design results that have been done, only pig DNA was amplified while the DNA of bovine chickens and dogs was not amplified. This results is also supported by the results of the negative control (NTC) containing Primary without DNA also not amplified. So that we can say that the primers of our design are specific only mopping to pig DNA.

The DNA used for further validation is fresh pork DNA for testing the linearity and repeatability stages. The linearity testing parameter is the linear relationship curve between Cq and the logarithmic function of DNA concentration, so that the correlation coefficient, y-intercept, and slope values will be obtained. The slope value is used to calculate efficiency. The sensitivity test was tested through 5 series of dilution of fresh dog meat DNA (5000, 1000, 500, 100 and 10 pg / μL).

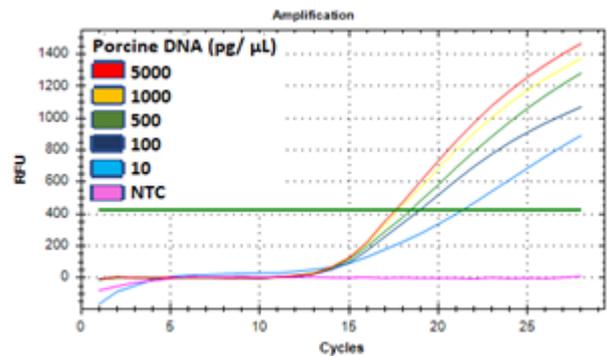
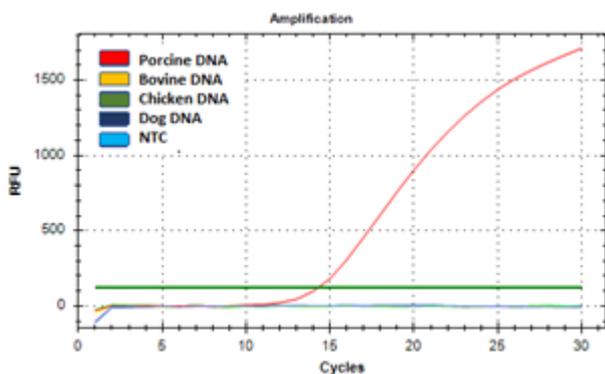


Fig. 3. Primary linearity test results from the fresh DNA dilution series of pork. (A) amplification curves and (b) standard curves

The results of amplification and linear regression curves are shown in Figure 3 and produce an equation with the value

of R2 0.908, slope -1.401, and y-intercept 22.254 gives good linearity. Efficiency (E) is given at 417.4%. R2 and efficiency values do not meet the recommended criteria ($R2 \geq 0.98$ and $E 90-110\%$) [17]. This results may be influenced by several factors, namely the inconsistent use of pipettes that can cause a low level of accuracy and the presence of inhibitors in the master mix [20]. A high E value indicates a disturbance in the target DNA that is duplicated by the intruder, causing template amplification in each cycle to be less exponential [21]. The repeatability test was carried out on fresh isolates of pig DNA with a concentration of 50 ng / μL to see the consistency of real-time PCR amplification results based on replication results. The Ct parameter used to determine the consistency of the method is the Coefficient of variation (CV) value. The CV value seen in Figure 4 meets the criteria recommended in the PCR method, which is $CV \leq 25\%$ [15]. The results of this repeatability amplification are also shown in Table 2.

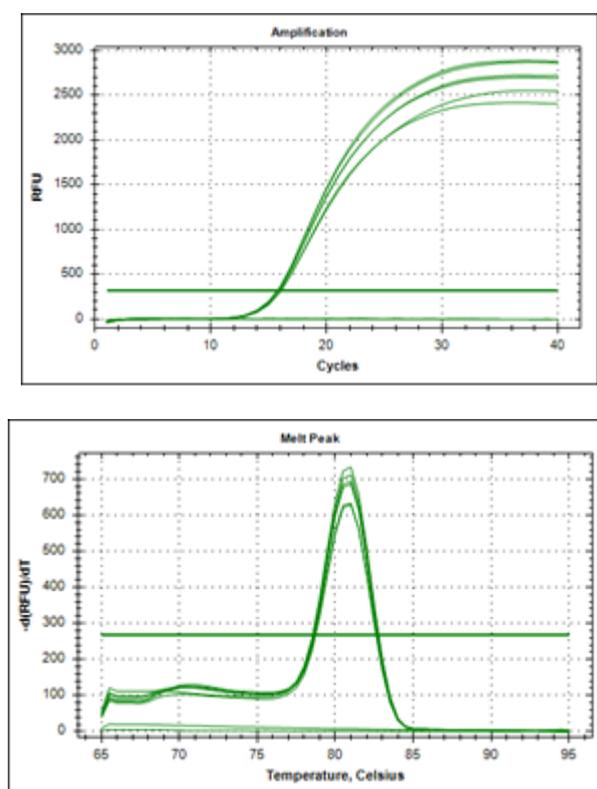


Fig. 4. The SYBR green binds to all types of double-stranded DNA

An increase in the number of amplicons in real-time PCR is recorded during the process by using fluorescent compounds as signal registers. The fluorescent compound commonly used in this study is SYBR green. SYBR green is a non-specific fluorescent dye that can undergo fluorescent processes under UV light and will increase if bound to a double strand of DNA. Dyes with SYBR green are said to be non-specific because they can detect all products that are not targeted and can form primary-dimers during the PCR process so that the Melting Curve Analysis (MCA) optimization needs to be done by increasing the temperature from 50-90oC. At that temperature, the product which is not targeted will be denatured at a lower temperature compared to the product targeted [22]. The SYBR green binds to all types of double-stranded DNA which will form the DNA-SYBR green complex and will emit light at a wavelength of 520 nm [11].

TABLE II. REPEATABILITY TEST RESULTS FOR FRESH PORK DNA (50 NG / μL)

Cq	Tm	average Cq	SD	The CV (%)
15.86	80.50	15.87	0.17	0.57
16,04	80.50			
15,70	80.50			
15,70	80.50			
15,87	80.50			
16,04	80.50			

Based on the results of the primary design and validation tests that have been carried out in this research, it has succeeded in designing a specific primer of pig DNA which means it can be used as a method for detecting counterfeit meat on the market.

IV. CONCLUSION

Primary pairs 5' - CGGAACAGACCTCGTAGAATG - 3'(forward) and 5' -GGTAATGATGAATGGCAGGATAA AG - 3' (reverse) from the pig mitochondrial region (Sus Scrofa) can specifically amplify the analysis of pig DNA on the DNA of cows, chickens and dogs. The real-time PCR method meets the validation criteria, and can be applied to identify pig DNA to detect counterfeit meat on the market.

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