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The Employment of Real-Time Polymerase Chain Reaction for the Identification of Bovine Gelatin in Gummy Candy

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ABSTRACT

Gelatin is widely used in food products, especially candy. Gelatin can be made by hydrolysis of skin and bone of bovine and pig, and the obtained gelatins are typically called as bovine gelatin (BG) and porcine gelatin (PG). The use of PG in food and pharmaceutical products is prohibited to muslim consumers, therefore, the availability of analytical method capable of distinguishing BG from PG is very urgent. This study aimed to identify BG in gummy candy products using specific primers with real-time polymerase chain reaction (RT-PCR) methods. The specific DNA primer was designed using PRIMERQUEST and NCBI software and subjected to primer-basic local alignment search tool (BLAST). The assessment of primer specificity has been carried out using DNAs extracted from PG and BG and those extracted from raw meats of pigs, rats, dogs, goats, cows, and chickens. RT-PCR method using primer targeting on mitochondrial Cytochrome B gene was applied to analyze candies purchased from the markets. The sensitivity test was performed by measuring amplification at six dilution series (10000, 5000, 1000, 500, 50, and 10 pg/ μ L) on BG-based candies. RT PCR using specific primer could detect DNAs within the samples containing BG at an optimum temperature of 55.2°C. The DNA detection limit was 500 pg/ μ L. The standard curve of the serial dilution of DNA isolate of BG produces the correlation coefficient (R-value) of 0.866 and an efficiency value (E) of 996.2%. Four products from the markets were examined using the designed primer. There from four samples were positive to contain BG. Real-time PCR using specific primer targeting on cytochrome-B (forward: 5'-ACTAGCCCTAGCCTTCTCTATC- 3' and reverse: 5'-TGTCAGTAGGCTGCTACTAGG-3') can be used for halal authentication analysis of gummy candy.

Keywords: primer mitochondrial cytochrome B gene, bovine DNA, gelatin, real-time PCR

INTRODUCTION

Gummy candy is one of Indonesia's favorite foods because it tastes sweet, and the texture is easy to consume. The main ingredient for making gummy candy is gelatin. Gelatin is a protein produced from the partial hydrolysis of collagen tissue extracted from the skin, connective tissue, and livestock bones, including fish and poultry (Sultana et al., 2018). Animals used in making

gelatin are pig, bovine, and fish (Rohman *et al.*, 2020). The most commonly used gelatins in food are bovine gelatin (BG) and porcine gelatin (PG). The use of PG is not allowed to be consumed by Muslim communities and considered as non-halal gelatin according to Scholar of Thought of *Safi'i*, while BG is allowable to be consumed and declared as halal gelatin (Rohman & Windarsih, 2020). Therefore, the identification of BG in food products

including gummy candy is very urgent for halal authentication analysis (Rohman et al., 2020).

Some analytical methods have been applied for analysis of gelatins including Fourier transform infrared (FTIR) spectroscopy (Cebi et al., 2016), liquid chromatography using fluorescence detection through amino acid composition previously derivatized using *ortho*-phaldehyde (Nemati et al., 2004) and derivate agent of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (Ismail et al., 2021), liquid chromatography-mass spectrometric (LC-MS/MS) (Huang et al., 2020), electrophoresis (Nur Azira et al., 2014), and DNA-based methods employing real-time polymerase chain reaction (PCR) (Sudjadi et al., 2016). Some of these methods are lack in specificity especially for analysis of gelatins in complex matrix, therefore, DNA-based methods are method of choice for analysis of gelatins. PCR has been successfully applied to detect pig species and pork mixtures, namely polymerase chain reaction (PCR) (Yilmaz et al., 2013) and Real-Time PCR (RT-PCR) (Lubis et al., 2018).

The real-time PCR method is the further development of conventional PCR methods. Although the conventional PCR method is easy to spell, the PCR results cannot provide quantitative information (Matsunaga et al., 1999). The real-time PCR method can answer the problems arising from conventional PCR because, Real-time PCR provides the amplification of the target sequence, and the amplification product can be directly observed in each cycle using a fluorescent-labeled probe (Erwanto et al., 2018). The fluorescence intensity is directly proportional to the amount of DNA reacting with fluorescent of evagreen (Jansson et al., 2017). Then the PCR products are analyzed using Melting Curve Analysis (MCA) without using agarose gel electrophoresis.

MATERIAL AND METHODS

Pork gelatin, beef gelatin, sodium bicarbonate, and trypsin (Sigma, Aldrich, USA). Beef, chicken, goat, and pork are obtained from traditional markets in Yogyakarta, while dogs and rats are obtained from the Ban area, Yogyakarta, Indonesia. DNA primers were designed and tested with software from the NCBI website and then ordered from Genetics Science Indonesia. The materials used for analysis and affect the accuracy of the pro-analysis quality test results (p.a).

Instrument

Real-time PCR CFX 96 (Biorad, USA), EFM 40 shaker (OSK, Seiwariko Co., Ltd), PharmaSpec UV-

1700 spectrophotometer (Shimadzu, Japan), vortex (Barnstead), and water bath (Haake L Fisons, Karlsruhe, Germany) and other glass tools commonly used in the analysis found at the Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada, Indonesia.

Methods

Primer design

Specific primers for bovine DNA used in this study were designed using the National Center Biotechnology Information (NCBI) Software (<http://ncbi.nlm.nih.gov/>) and designed *in silico* using the NCBI-BLAST website from NCBI (<http://blast.ncbi.nlm.nih.gov/>) to see the specificity of the primers obtained.

The preparation of Reference candy

The Gummy candy samples were prepared according to Pratiwi et al. (2018). First, 20.0g of gelatin were weighed and soaked in 100mL of water for 15min. Then, 150.0g of granulated sugar and 5mL of dye/ flavor are dissolved in 100mL of water. The solution of sugar and flavoring is then poured into the gelatin bath and cooked until it boils while continuously stirring. When the dough has thickened, then pour it into the mold and wait for it to solidify. After that, the candies are sprinkled with sugar so they don't stick to each other.

Sample Preparation

For gelatin standards, the sample preparation was carried out according to Cheng et al. (2012). First, as much as 100mg of standard gelatines was dissolved in 50mL of ammonium bicarbonate solution (1% NH_4HCO_3 , pH 8.0). The solution was filtered using a 0.22 μm syringe filter. Furthermore, 100 μL of gelatin solution was taken and added with 10 μL of trypsin solution (1mg/mL in 1% NH_4HCO_3 , pH 8.0). The solution was incubated at 37°C for 12h).

For gummy candy, the sample preparation is performed according to Tan and Lock (2014). Each sample (5mg) has dissolved in 600 μL of ammonium bicarbonate buffer solution (37°C within 10-15min). This extract has digested using trypsin (50mM in ammonium bicarbonate buffer with trypsin ration: sample (1:100). This solution has incubated at 37°C for 12h. The digested sample has centrifuged at a speed of 12,000 rpm for 5. The upper supernatant layer (500 μL) was taken care of without disturbing the lower sediment. The supernatant is again centrifuged at 12,000 rpm for

5min. The top supernatant layer (200µL) was used for analysis by PCR.

DNA isolation

DNA isolation methods from gelatin or gummy candy made from pork and beef gelatin were carried out with Mitochondria DNA Isolation Kit from Biovision (USA).

The purity of the isolated DNA was analyzed by measuring the absorption of 20 µL DNA solution (in 980µL aquabidest) at wavelengths of 260nm and 280nm. The concentration of DNA isolated was calculated from the absorbance at wavelengths of 260nm (A260) multiplied by the dilution factor and the absorption constant (50ug/mL) (Lee et al., 2014).

Real-Time PCR Analysis

The reaction mixture (20µL) has used for real-time PCR Analysis. It consists: 10µL evagreen universal PCR master mix; 1µL forward and reverse primer, 1µL DNA template (50ng/µL), and 7µL nuclease-free water. Optimization of the primer's temperature that has been designed for pig DNA was carried out in a temperature range of 52 to 62°C using real-time PCR (Bio-Rad, 2006). The validation parameters evaluated included primer the specificity testing of primers, linearity test, and determination of detection limits (Orbayinah et al., 2019).

Analysis of commercial gummy candy

Analysis of commercial gummy candy samples were carried out using four kinds of samples available in Indonesia and not yet labeled halal. The analysis steps included DNA isolation, measurement of DNA purity and concentration, as well as DNA analysis of market gummy candy samples using real-time PCR.

RESULT AND DISCUSSION

The primer design of primers refer to the procedure carried out by Cai et al. (2020) designing *Bos taurus* primer using a template with an accession number AF492351. The obtained primers were forward: 5'-ACTAGCCCTAGCCTTCTATC-3' and reverse: 5'-TGTCAGTAGGCTGCTACTAGG-3'. The first step is to optimize the annealing temperature (Ta) of the primers, capable of amplifying the target sequence specifically. The requirements for selecting Ta are determined based on a high RFU value and a low number of cycles. Temperature of the primer attachment is obtained using varying quantitation cycle (Cq) values, where the

amplification of bovine DNA at a temperature of 55.2°C gives the highest amplification response with an RFU value of 1000 and a low Ct value (26.07) with a value Tm 82.50°C (Figure 1).

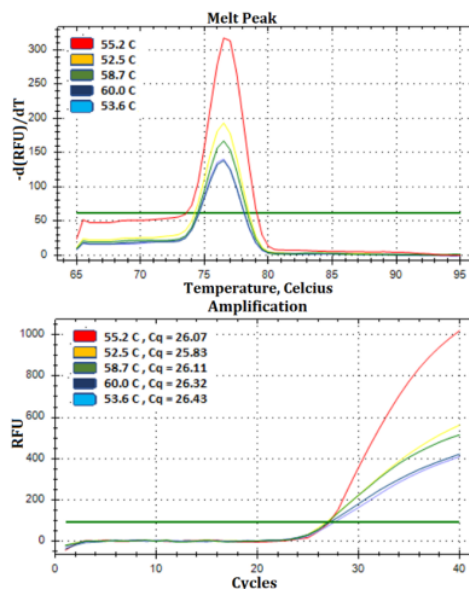


Figure 1. Amplification of designed primers of Primer using different annealing temperature (Ta) to Bovine DNA using Real-Time PCR.

Furthermore, a temperature of 55.2°C was used to test the specificity of designed primers to other DNAs from different species, namely Bovine (*Bos taurus*), porcine (*Sus scrofa domestica*), Chicken (*Gallus gallus*), dogs (*Canis lupus familiaris*), goats (*Capra hircus*) and rat (*Rattus argentiventer e*). The designed Primers revealed good specificity against various other DNAs (Figure 2).

The primer specificity is an absolute requirement during testing with Real-Time PCR method in order to amplify target species among other species. The primer amplification results in the target area need to be verified for the order of the bases to prove the specificity of primers. The purpose of verification is to ensure that the PCR amplification results with specific primers result from amplification in the target area, not another site in the same species or another site in a different species. Amplification results of the primer specificity test on the DNA of other species show that only the DNA of the target species provides an

amplification response for bovine (*Bos taurus*) (Figure 2). In contrast, the DNA of non-target species does not show an amplification response in porcine (*Sus scrofa domestica*), chicken (*Gallus gallus*), Dogs (*Canis lupus familiaris*), goats (*Capra hircus*), and rat (*Rattus argentiventer e*). This means that the primers used are specific only to bovine DNA. This specificity test was also carried out on the gelatin DNA of bovine, porcine, and reference candy made from bovine gelatin (Figure 3).

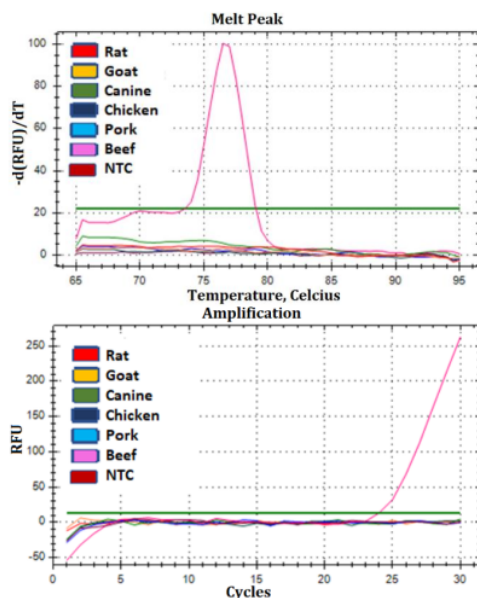


Figure 2. Amplification curve of the specificity test results against five other DNA species and control (NTC) using real-time PCR.

Based on these results, it can be seen that the designed primers can amplify DNA originating from cow gelatin only, while pig DNA and negative controls that do not contain DNA are not amplified. This makes it even clearer that the primers are really specific only to the DNA of cows, whether it comes from meat or gelatin.

Primers that have been declared specific at their optimum T_a (55.2°C) are then used for the validation of the real-time PCR method for the analysis bovine DNA. The parameters evaluated include sensitivity test, detection limit test, and repeatability test. During the validation test, DNA isolate from standard bovine Gelatin was used.

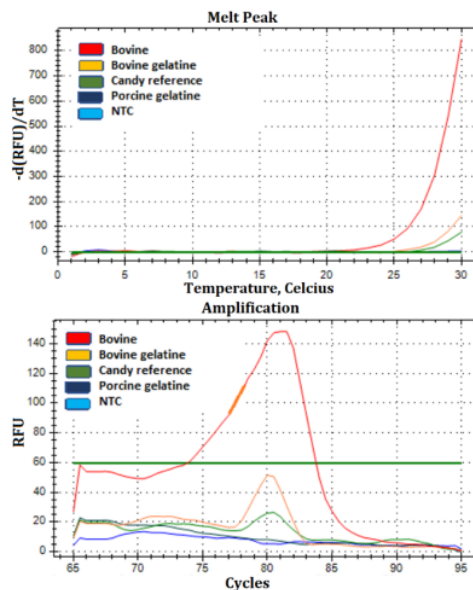


Figure 3. The Amplification Curves during the evaluation of Primer Specificity Test Against Gelatin, Reference and Control Candy (NTC) Using Real-Time PCR.

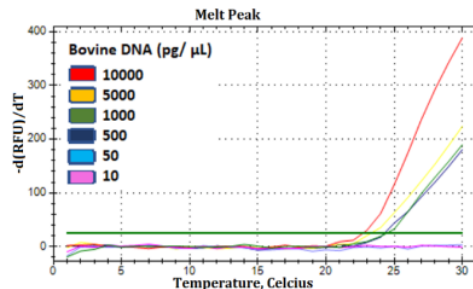


Figure 4. The Amplification curve of DNA isolate extracted from standard bovine gelatin in the dilution series

The sensitivity test aims to determine the smallest concentration (nanalytes (gelatin DNA) that can still be amplified using Real-Time PCR. The sensitivity test was carried out by making six series of dilutions (10000; 5000; 1000; 500; 50; and 10pg/μL) of DNA isolates extracted from the standard bovine gelatin (Figure 4).

The Amplification results of the bovine gelatin DNA isolate show that the smallest measurable concentration is 500 pg, while the concentrations of 50 and 10 pg cannot be measured (Figure 4). This may be due to insufficient DNA from the raw material for gelatin, which may have been damaged during the processing into gelatin, which requires high heat, so it requires a larger cycle to be amplified.

The measurement of sensitivity⁷ is represented by the intersection curve of the linear relationship between Ct (Cycle threshold) and the Logarithm of DNA concentration. Based on the intersection results, the correlation coefficient R, intercept, and slope was obtained. The slope value is then used to determine the amplification efficiency. Based on the amplification data series dilution of pork and wild boar DNA isolates, a relationship curve between Ct and the logarithm of DNA concentration was made (Figure 5).

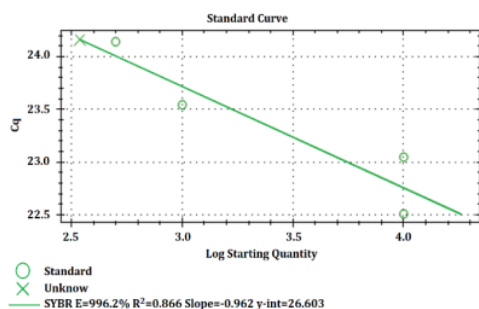


Figure 5. The raw curve of the dilution series of bovine gelatin DNA isolates

Based on Figure 5, the standard curve of the serial dilution of the bovine gelatin DNA isolate produces the correlation coefficient (R-value) of 0.866 and an efficiency value (E) of 996.2%. The resulting value (r) doesn't meet the acceptance criteria set at a minimum of 0.98, and the resulted E value also meets the required acceptance criteria, namely 90-105% (Rohman et al., 2017). The resulting E value is influenced by several factors, namely the pipetting's accuracy during preparation and the presence of inhibitors in the master mix used. The E value that is close to 100% indicates good repeatability and proximity of the amplification value to the amplification's theoretical value (Widyasari et al., 2015). The high E value suggests that there is a disturbance during the target DNA copy process so that the number of DNA copies produced per cycle does not follow the

2n rule. In contrast, the low E value is due to the less than optimal thermocycler condition, so that the primer attachment is not perfect (Hossain et al., 2019).

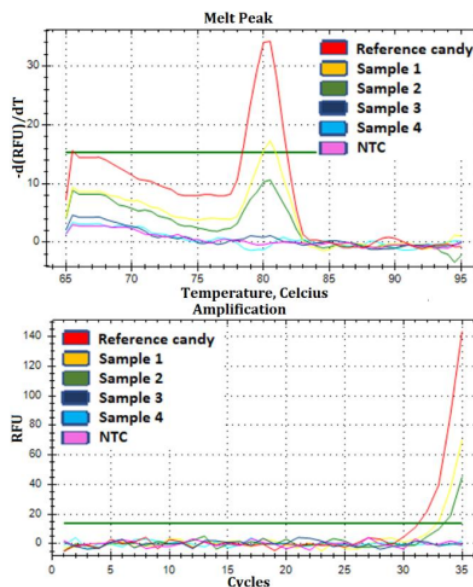


Figure 6. Amplification curve of market soft candy DNA isolates

Analysis of commercial gummy candy samples was carried out to identify gelatin DNA in gummy candy sold in several supermarkets in Yogyakarta and Jakarta. The identification of DNA gummy candy in the market is carried out using the same procedure as the Identification of pure gelatin DNA and reference DNA gummy candy. It started from the DNA isolation, qualitative testing, and quantitative testing of the results of DNA isolates and sample testing with Real-Time PCR. Figure 6 shows the qualitative test results of market DNA gummy candy isolates. Based on the amplification results of 4 marketed samples without halal label, it turns out that cow DNA primers do not apply two candy samples, therefore, it can be deduced that the gelatins used in the two soft candies is not prepared from bovine gelatin as raw material.

CONCLUSION

The Real-time PCR combined the species-specific primer targetting on cytochrome b *Bos taurus* (forward: 5'-ACTAGCCCTAGCCTTCTCTATC-3' and reverse: 5'-

TGTCAGTAGGCTGCTACTAGG-3'). can be used for the Identification of bovine DNA from gelatin. The developed method is specific and reliable for the identification of gelatin sources in food and pharmaceutical methods. In the future, this method can be standardized through collaborative studies and can be routinely applied for halal authentication analysis.

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