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Real-Time Polymerase Chain Reaction for Halal Authentication of Gelatin in Soft Candy

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ABSTRACT

Currently, along with the development of science and technology, the diversification of food products occurs in the market. Food products can contain non-halal components like porcine gelatine. One of food suspected to use gelatine is soft candy. Gelatin can be made from pork or beef or other animal. The presence of porcine gelatine in any food products is not allowed for Moslem community, therefore an analytical method offering reliable results must be developed. This study is intended to use Real-Time Polymerase Chain Reaction (RT-PCR) for analysis of porcine gelatine in soft candy. Isolation of DNA was performed with mitochondrial DNA Isolation Kit K280-50 (Bio-Vision). The DNA was analyzed by RT-PCR using primer D-Loop 318. Analysis for the primer specificity was performed on fresh tissue (pig, cows, chickens, goats and rats) and gelatin sources (beef, pigs and catfish). Primer D-loop318 can amplify porcine DNA at the optimum temperature 61.4°C. Repeatability test demonstrated amplification of all positive response samples containing porcine DNA in serial dilution of 10000-1 pg). The Coefficient of Variation (CV) is 6.32%. The repeatability test was also performed on soft candy 100% having CV of 1.06%. The commercial soft candy samples evaluated do not contain porcine DNA.

Key words: Polymerase chain reaction, porcine gelatine, soft candy, halal

INTRODUCTION

Today, due to the development of science and technology, the diversification of food products is available in the market. As a consequence, food products can use non-halal components to reduce production cost. In the market, porcine gelatine is cheaper that bovine gelatine or other gelatine produced from halal sources (Widyaninggar *et al*., 2012). Any products containing pig derivatives such as porcine gelatin is not allowed to be consumed according to some Islamic scholar, indeed, the tools to detect the presence of porcine gelatin is necessary to assure the halalness of certain products (Rohman and Man, 2012).

Chemically, gelatin is a mixture of polypeptides prepared by hydrolysis of collagen. Gelatin can be extracted from skins, bones and hides of mammalian animals such as pig and beef (Karim and Bhat, 2008). Besides, gelatine can also prepared from fish (Norziah *et al*., 2009; Gimenez *et al*., 2005; Kolodziejska *et al*., 2004). According to GMIA (2012), commercial gelatin is obtained from bovine and porcine, in which an approximately of 90% of gelatin is coming from porcine. Gelatine has gelling properties such as gel strength and gelling time, setting and melting temperature and

viscosity which is suitable to be used in food products such as soft candy. Besides, the surface behavior of gelatin (e.g., formation and stabilization of foams and emulsions, adhesive properties and dissolution behavior) have justified its use in food products (Schrieber and Gareis, 2007; Azira *et al*., 2014).

Several reports have been published with respect to analytical methods capable of distinguishing porcine and bovine gelatines. Such methods are infrared spectroscopy coupled with chemometrics of Principal Component Analysis (PCA) for differentiation of porcine and bovine gelatins (Hashim *et al*., 2010) and those with fish gelatine (Cebi *et al*., 2016), high performance liquid chromatography coupled with fluorescence detector and chemometrics of PCA (Nemati *et al*., 2004; Raraswati *et al*., 2013) and with some types of mass-spectrometer detectors (Zhang *et al*., 2009; Yilmaz *et al*., 2013), electrophoretic analysis (Hermanto *et al*., 2013), Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) combined with PCA (Azira *et al*., 2014), Enzyme-Linked Immuno-Sorbent Assay (ELISA) (Doi *et al*., 2009; Venien and Levieux, 2005), conventional method using calcium phosphate precipitation test (Hidaka and Liu, 2003) and Polymerase Chain Reaction (PCR) (Demirhan *et al*., 2012; Cai *et al*., 2012). The PCR is an ideal technique to be used for fat and sensitive detection of porcine DNA in gelatin due to the higher stability of DNA compared to protein (Aida *et al*., 2007). With the development of Real-time PCR offering sensitive and specific enough to trace small amounts of target DNA. This technique becomes popular tool to detection of bovine and porcine DNA in gelatin mixtures, gelatin-containing food products and capsule shells (Shabani *et al*., 2015).

In this study, two sets of new primers were designed using Primer NCBI-BLAST software at the NCBI website Primer-BLAST was evaluated. Two primer pairs used, i.e. D-loop 340 and D-loop 318, are evaluated to amplify DNA from porcine gelatin specifically in commercial soft candy. Furthermore, the specific primer is subjected to validation step by determining specificity, sensitivity, linearity and repeatability. Finally, real-time PCR using the designed primers is used for analysis of commercial soft candy.

MATERIALS AND METHOD

Porcine gelatin and bovine gelatines were purchased from Sigma-Aldrich (St. Louis, MO). The commercial soft candy were purchased from several markets in Yogyakarta, Indonesia. Spectrophotometer UV-Vis UV-1700 PharmaSpec (Shidmadzu, Japan) was used for DNA quantification. Realtime PCR CFX 96 (Biorad, USA) was used for PCR amplification, while electrophoresis (i-Mupid J Cosmo Bio Co, Tokyo, Japan), mini and transluminator (Biorad, USA) are used for DNA identification. This study is conducted during March-December 2014.

Oligonucleotide primers: The oligonucleotide primers targeting mitochondria D-loop were designed using Primer NCBI-BLAST software at the NCBI website Primer-BLAST (Table 1). All primers were obtained from PT Genetika Science Indonesia (Jakarta, Indonesia).

Preparation of soft candy: Preparation of soft candy was carried out according to Raraswati *et al.* (2013) with slight modification. Briefly, an approximately 20 g of bovine gelatine or porcine gelatine were weighed quantitatively and subsequently immersed with 100 mL of water

Table 1: Oligonucleotide primers used for detection of porcine gelatine in soft candy samples

Template	Sekuen	Tm (°C)	GC (%)	Product length
D-loop 340	Forward: TGCATTAACTGCTAGTCCCCA	59.09	47.62	168
	Reverse: TTTCACGCGGCATGGTAGTT	60.60	50.00	
D-loop 318	Forward: TGCATTAACTGCTAGTCCCCA	59.09	47.62	146
	Reverse: GCTCGTGATCTAGTGGTGGT	59.18	55.00	

Tm: Melting temperature

for 15 min. Meanwhile, 150 g of sugar and 3 mL of fruit flavor were dissolved in 100 mL water. Gelatin immersed was subsequently poured into a pan containing solution of sugar and fruit flavor. The solution was cooked and stirred constantly until all of the gelatins were soluble and thickened. The solution was subsequently removed from the heat and poured into the prepared loaf pan. The solution was stand for 4 h to obtain a smooth and chewy texture. The candy was firmly cut and then dips them in powdered sugar. The mixture of porcine-bovine gelatines is made a series concentration levels of 0, 10, 20, 30, 40, 50 and 100% (w/w) of porcine gelatine.

DNA isolation from gelatin and soft candy containing gelatine: Isolation of DNA was performed by DNA isolation KitK280-50 according to manufacturer instruction (BioVision Inc., 2008). Briefly, an approximately of 3 g of soft candy was transferred into conical tube15 mL, mixed with phosphate buffer saline 2 mL and incubated at 65°C for 60 min. One milliliter of this solution was pipetted into a 2 mL clean tube, added with 1 mL of 1X cytosol extraction buffer, shaken and incubated for 10 min. The mixture was centrifuged at 10000×g for 10 min and subjected to further centrifugation at 15,000×g for 30 min. The supernatant is discarded and eluate was added with 1 mL of 1X cytosol extraction buffer and centrifuged again at 15,000×g (4°C) for 30 min. The supernatant is discarded and eluate was added with 30 μL of mitochondrial lysis buffer, 25 μL of enzyme B mix and incubated at water bath 50°C for 60 min. The eluate was added with 100 μL of absolute ethanol, stored at -20°C for 10 min. Subsequently, the eluate was centrifuged at 15,000×g for 5 min. The supernatant is discarded and eluate was washed twice using 1 mL of 70% cold ethanol. The precipitate was air dried for ±5 min, added with 40 μL of buffer TE and stored at -20°C until being used for analysis.

PCR amplification: Amplification of DNA using primers of D-loop 340 and D-loop 318 was performed in a final volume of 20 μL, containing of 10 μL of SYBR Green master mix, 1 μL of forward primer and 1 μL of reverse primer, 4 μL of DNA template (50 ng) and water free RNA-ase. The condition of DNA amplification assay consisting of initial denaturation at 95°C for 15 sec, annealing at an optimum temperature and elongation at 72°C for 10 sec. The amplification products were electrophoresed through on agarose 0.8% stained with ethidium bromide, according to Sambrook *et al.* (1989).

Determination of the sensitivity and repeatability of the assay: The determination of sensitivity assay of primers D-loop 340 and D-loop 318 was expressed as detection limit of porcine DNA in pure gelatine and in soft candy. The replicate of real-time PCR measurements was made of dilution series of (1000, 200, 100, 10, 5 and 1 pg μL^{-1}) porcine gelatin and soft candy containing porcine DNA. The Limit of Detection (LoD) was taken as being the lowest amount that could be amplified with a reproducible Ct value. A similar approach was adopted to determine LoD in porcine gelatine spiked into soft candy samples. The repeatability assay was performed by replication of these dilution series in three replicates.

RESULT AND DISCUSSION

In this study, we examine the presence of porcine DNA in soft candy, a favorite food for children using real-time polymerase chain reaction (Real-time PCR). The primers used was targeted on mitochondrial the D-Loop region (D-loop 340 and D-loop 318). The primers of D-loop 340 and

Fig. 1: Electrophoretic results of DNA from soft candy containing porcine and bovine gelatines. Lane A: Porcine-bovine 100: 0%, Lane B: Porcine-bovine 50: 50%, Lane C: Porcine-bovine 40: 60%, Lane D: Porcine-bovine 30: 70%, (E) Porcine-bovine 20: 80% and Lane F: Porcine-bovine 10: 90%

D-loop 318 revealed that bases G or C in last 5 position of the 3' end are less than 3. These can increase the specific binding at the 3' (Van Pelt-Verkuil *et al*., 2008). Besides, it does not form GC clamp folds (IkaWidyasari *et al*., 2015). In addition, the amplicon length less than 250 bp can increase the efficiency of PCR method (Wang and Seed, 2006).

Isolation of DNA was performed by DNA isolation KitK280-50. Isolation of DNA is intended to separate DNA from the cell matrix and other components in the cell. The process of DNA isolation was performed through several stages, namely destruction of cell membranes (lysis), process of DNA extraction using organic solvents, purification, precipitation and concentration (Sambrook *et al*., 1989). The isolated DNA from pure porcine gelatin and soft candy was qualitatively analyzed using gel electrophoresis 0.8% agarose. As indicated in Fig. 1, DNA was present without any contamination from RNA. The presence of RNA can interfere PCR amplification process. The DNA concentration and its purity were measured using spectrophotometer UV at λ 260 and 280 nm. The concentration of DNA obtained is in the range of 10-1075 μg mL^{-1}.

During PCR analysis, the designed primers are optimized in order to determine appropriate annealing temperature at range 52-62°C and the number of cycles is limited to 35. Primer D-loop 340 showed amplification either the porcine and bovine DNA and have two peaks on melt peak curve (Fig. 2), while the primer D-loop 318 can amplify porcine DNA at the optimum temperature 61.4°C (Fig. 3). At this temperature, porcine DNA is amplified with low number of

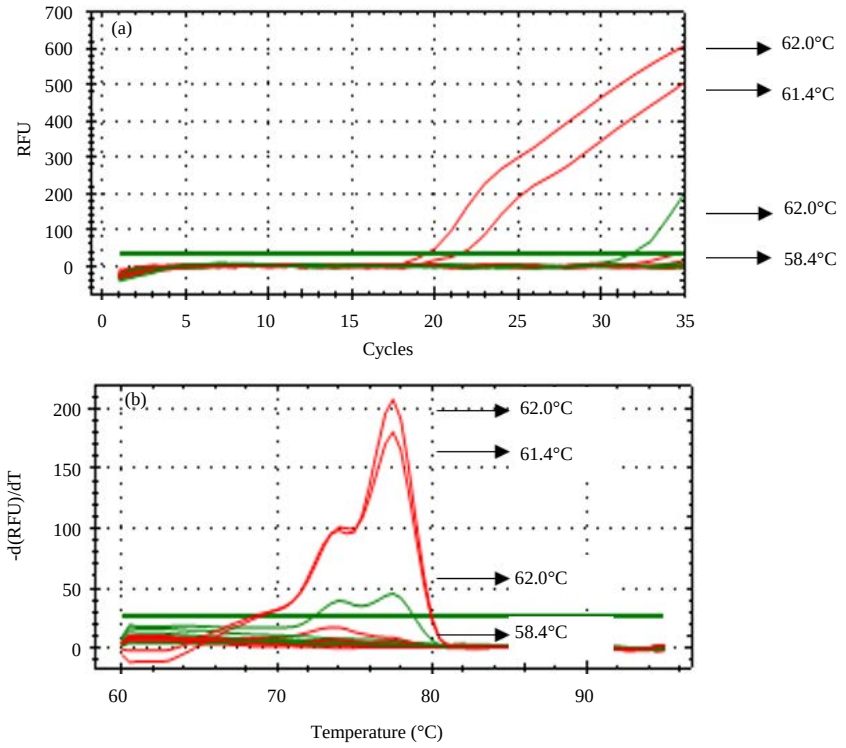

Fig. 2:(a-b): (a) Amplification curve of porcine and bovine DNA using primer D-Loop 340 at different annealing temperature and (b) Melting curve analysis of during amplification of porcine and bovine DNA using primer D-Loop 340. Red: Porcine DNA, Green: Bovine DNA

cycles, have one peak and highest Relative Fluorescence Unit (RFU) value. Therefore primer D-Loop 318 was chosen for further analysis. The selected primer (D-loop 318) was subjected to specificity test toward DNA from fresh tissue of animals (pig, cows, chickens, goats and rats) and gelatin sources (beef, pork and catfish). Amplification was also performed on prepared soft candy containing porcine-and bovine gelatins. Primer D-loop only amplify porcine DNA and do not amplify other DNA, as shown in Fig. 4 and 5.

The sensitivity of real-time PCR using D-loop 318 was expressed by Limit of Detection (LoD). For determination of LoD, dilution series (10000, 1000, 100, 10, 5 and 1 pg) are used. Porcine DNA can still be amplified up to 10 pg, while at 5 pg, porcine gelatin DNA is not amplified to cycle of 35, therefore it is judged that LoD value of DNA to be amplified is 10 pg. The R^2 obtained for the relationship between log of DNA concentration (x-axis) and cycle threshold (Ct) was 0.980, with y-intercept of 35.83. The amplification efficiency (E) is 262.1% (Fig. 6).

Some factors can affect the value of E, namely the assay performance depending on the primers' and template sequences and structures, the sample matrix containing inhibitors and other interfering substances from the sample or carry overs agents from upstream processing steps, the type of reagents and its concentrations used and the presence of competing reactions (Svec *et al*., 2015). These results exceeds the criteria in Bio-Rad (2006), which are 0.980 and 90-105% for R^2 and E, respectively. The unrealistic of E (E = 262.1%, E>100%) can be caused by inhibitors present in

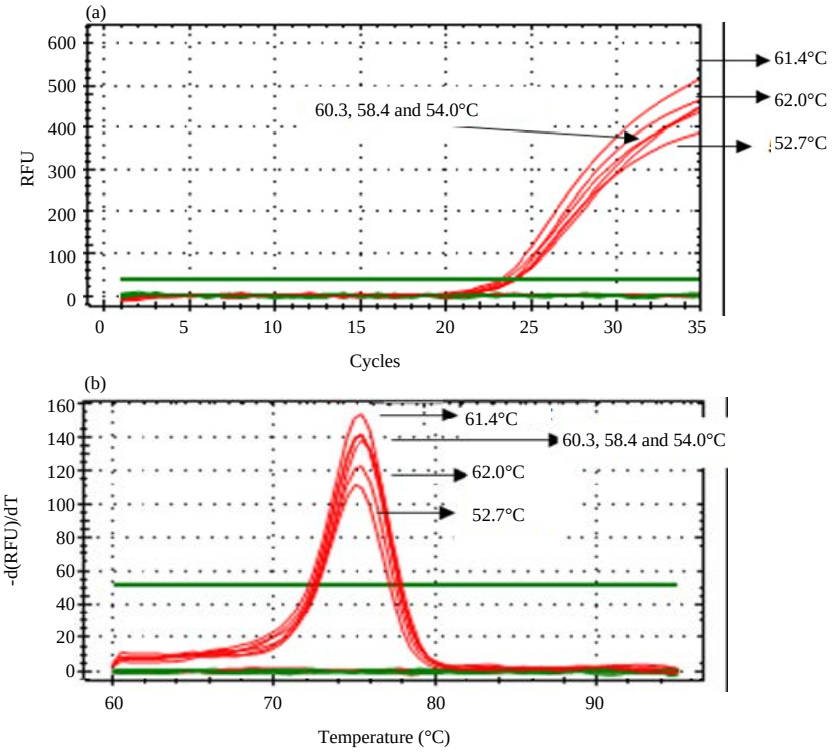

Fig. 3(a-b): (a) Amplification curve of porcine and bovine DNA using primer D-Loop 318 at different annealing temperature and (b) Melting curve analysis of during amplification of porcine and bovine DNA using primer D-Loop 318. Red: Porcine DNA, Green: Bovine DNA

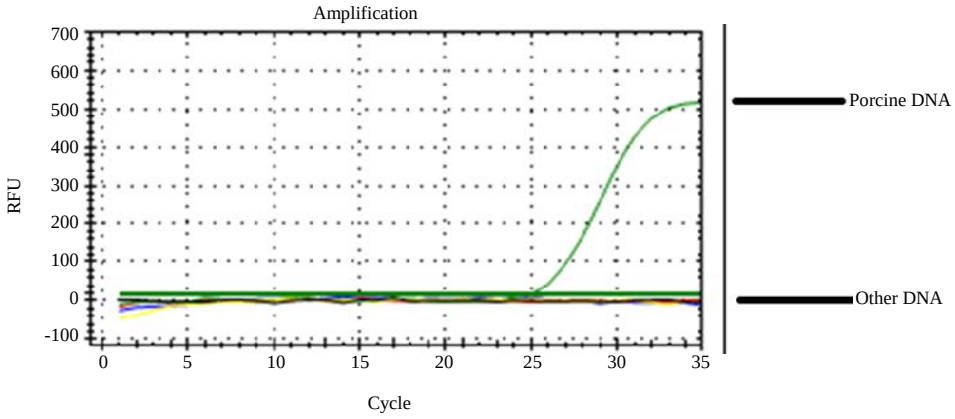

Fig. 4: Amplification of porcine DNA using primer D-loop 318

the mixture with high concentration. Standard curve were also obtained from porcine-bovine gelatin soft candies (0, 10, 20, 30, 40, 50 and 100%). The R^2 obtained is 0.910 and E = 64.0%. The low value of E can be caused by lack of pipetting precision and DNA extraction methods (Muhammed *et al*., 2015).

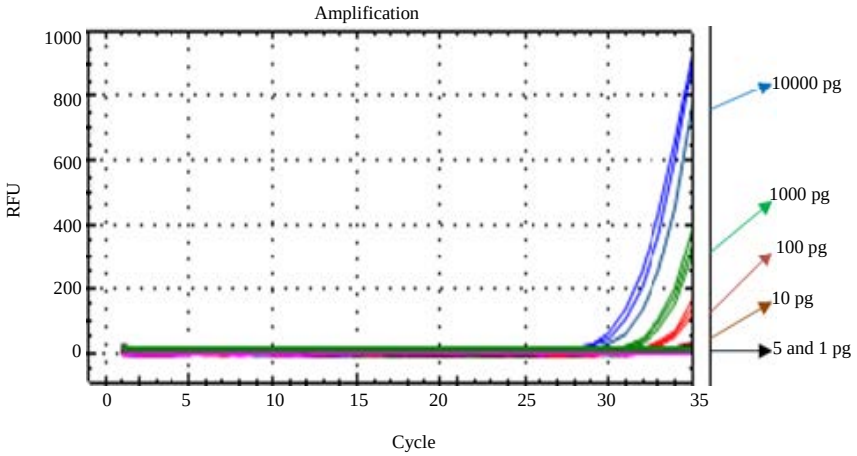

Fig. 5: Amplification of porcine DNA using primer D-loop 318 as function of porcine DNA concentration

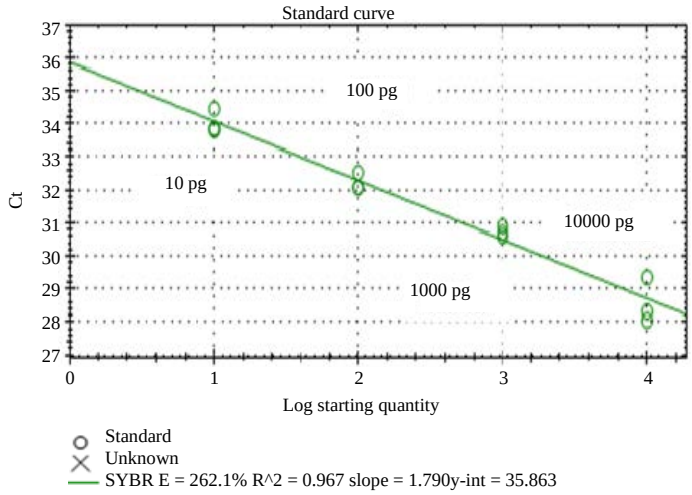

Fig. 6: Relationship between log of DNA concentration (x-axis) and cycle threshold (Ct) of porcine DNA using primer D-loop 318

Repeatability test demonstrated the amplification of all positive response samples containing porcine DNA in serial dilution (10000-1 pg). The Coefficient of Variation (CV) of 6.32%, which was lower than that of CV maximum allowed for PCR analysis, i.e., ≤25%, according to requirement stated in Codex Alimentarius Comission (CAC., 2010). Repeatability test was also performed on soft candy 100%. The Coefficient of Variation (CV) of 1.06% was obtained. The primer D-loop along with real-time PCR analysis was subsequently used for identification of porcine gelatin DNA in commercial soft candy samples. No amplification is found in the commercial samples. This demonstrated that commercial soft candy samples do not contain porcine gelatin DNA (Fig. 7).

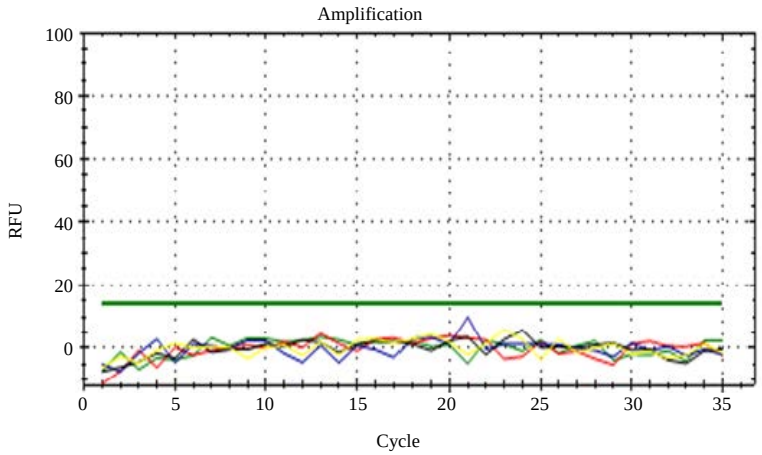

Fig. 7: Amplification of DNA extracted from commercial samples of soft candy obtained from some local markets in Yogyakarta. No amplification is found for all samples tested

CONCLUSION

Primer D-Loop 318 with a length of amplicons 146 bp is specifically able to identify the presence of porcine DNA in fresh tissue and gelatin sources at optimum annealing temperature of 61.4°C. The limit of detection of porcine DNA was 10 pg. The Coefficient of Variation (CV) on repeatability analysis was 6.32%. Five products from market were examined. No amplification is found among samples tested, meaning that soft candy samples do not contain porcine gelatin.

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