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Application of real-time polymerase chain reaction for analysis of porcine DNA in gelatine-containing capsule shell for halal authentication

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Abstract

Gelatine used for capsule shells is made from porcine or bovine origins. Several methods for porcine DNA identification in numerous products have been reported for halal authentication. The negative results of a specific determination toward porcine DNA remained unclear, whether negative results were caused no porcine DNA in product or due to the failure during DNA extraction, therefore it is necessary to confirm the presence of bovine gelatine in capsule shell. The aim of this study is to confirm the bovine gelatine using specific primer from bovine D-loop. From two pair of primers designed, only primer D-loop93 (F: ACACAGAATTTGCACCCTAA, R: GTACATTACCCCTTGCGTAG) had the capability to identify bovine DNA either in fresh tissue or gelatine sources with the optimum annealing temperature of 51.4^{o}C. The limit of detection of DNA in gelatine is 5 pg. All commercial capsule shells were analyzed using primer D-loop 93, and the results showed that all commercial capsule shells are amplified.

Introduction

Gelatine is soluble protein obtained by partial hydrolysis of various sources of collagen such as bones, skins, and cartilages of pig and cattle. The most abundant sources of gelatine are pig skin (46%), bovine skin (29.4%), as well as pig and cattle bones (23.1%) (Gomez-Guillen *et al*., 2009). Currently, some other sources of gelatin are also developed like fish gelatin. Gelatine is an essential hydrocolloid material used in some application, including food, cosmetics and pharmaceutical products, because of its gelling and thickening properties. Gelatine is composed from all essential amino acids needed by human body except tryptophan (Ladislaus *et al*., 2007). In the pharmaceutical industry, gelatine is used for making hard and soft capsule shell (capshells), granulation, tableting, coating tablet, vaccines excipients, and encapsulation. Besides, gelatine can prevent the oxidation and make products more palatable (Gibbs *et al*., 1999). Gelatine applications have increased in food and pharmaceutical industries since the encapsulated materials can be protected from moisture, heat or other extreme conditions in order to enhance its stability and maintaining viability (Gibbs *et al*., 1999).

In the market, 90% gelatine comes from porcine gelatine, because porcine gelatine is cheaper than other sources of gelatine (Widyaninggar *et al*., 2012). Nevertheless, Muslims are forbidden to consume any pig produtcs due to God's rule in Al-Qur'an as appear in surah Al-Maidah: 3 (Nurdeng, 2009). Alongside, Hindus are also prohibited to consume cow and its products, and there are some patients having gelatine allergic, in particular gelatine's source (Venien and Levieux, 2005a; Doi *et al*., 2009). Consequently, distingushing between porcine and bovine gelatines in all products is highly required, especially in pharmaceutical products.

Numerous methods based on physico-chemical and molecular biology have been reported to differentiate gelatine's source. Nemati *et al.* (2004) differentiated bovine and porcine gelatines based on amino acid profiles, as analyzed using reversed phase-high performance liquid chromatography in combination with fluorescence detection, while Hidaka and Liu (2002) used pH drop method after calcium phosphate precipitation. Zhang *et al*. (2009) have identified specific marker peptides in tryptic-digested bovine and procine gelatine by HPLC-MS/MS. Vinien and Levieux (2005b) developed indirect ELISA using synthetic bovine's specific antibodies by immunization of rabbits. Nowadays, the biological methods based on chain reaction on specific DNA sequence amplification is the most reported ones (Cai *et al*., 2012; Demirhan *et al*., 2012).

Real-time polymerase cahin reaction (real-time PCR) is method of choice for confirmation and

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differentiation of species and its products. Sudjadi *et al.* (2016) have differentiated between bovine and porcine gelatines using real time-PCR with new specific primer for D-loop sequence of porcine in capsule shell. However, results of those methods still contain possibility to be false negative of recognizing porcine gelatine. Almost the published works regarding the halal authentication are analysis or identification of porcine gelatin, even the negative results may come from no porcine DNA in product or due to the failure during DNA extraction. Therefore, it is a need to confirm that gelatine used is coming from bovine gelatine to assure the halal status of bovine gelatin. The aim of this study is to confirm bovine gelatin in chapsells using RT-PCR with newly designed specific primer for D-loop sequence of bovine.

Materials and Methods

Porcine and bovine gelatines were purchased from Sigma-Aldrich (St. Louis, MO, USA). The commercial capsule shell were purchased from several producers in Indonesia. Spectrophotometer UV-Vis PharmaSpec UV-1700 (Shidmadzu, Japan) was used for quantifying DNA. Real time PCR CFX 96 (Biorad, USA) was used for PCR amplification, and vortex (Barnstead, USA), shaker EFM-60 (Seiwa riko, Japan), oven UN75 (Memmert, Germany), waterbath WNB 7-45 (Memmert, Germany), microsentrifuge Sartorius 3-30K (Sigma) are used in DNA extraction.

Oligonucleotide primers

Primers were designed using feature pick primer from online software of NCBI website, which targeted on mitochondria D-loop of bovine (Table 1). All primers are bought from PT Integrated DNA Technology (IDT) (Jakarta, Indonesia).

Preparation of capsule shell

The capsule shells are prepared according to Widyaninggar et al. (2012), briefly an approximately of 5 g gelatine (i.e bovine and porcine) was moistened with 10 mL warm water. Subsequently, the mixture is added with 2 mL glycerin and 2 drops of food coloring agents with continuous stirring above hot plate untill clear batter appear. The mixture is immediately poured into dish and left to cool in ambient temperature. The batter of bovine-porcine gelatine is used to make a series of concentration of 0, 50, 60, 70, 80, 90, and 100% (w/w) of bovine gelatine.

DNA extraction from gelatine and capsule shell containing gelatine

DNA extraction was performed by phenol-chloroform-isoamyl alcohol (25:24:1 v/v/v) method according to Sambrook *et al.* (1989) with slight modification. Approximately of 0.2 g sampel (i.e gelatine or capshells) was immersed in PBS 20x – ethanol absolute 1:1 (i.e preparation solution) and was incubated in waterbath at 65°C till all samples dissolved. Subsequently, the mixture was added with absolute ethanol before centrifuging it at 13000 rpm for 3 minutes. The supernatant was then added with 800 µL lysis buffer and 20 µL proteinase K for each sampel, and was incubated in waterbath 65°C for 75 minutes with occasionally vortexed. After that, it was added with 10 µL RNAse and incubated in waterbath 38°C for 30 min. Each sampel was added with phenol 0.5x volume and chloroform-isoamyl alcohol 0.5x volume, shaken for 40 minutes and centrifuged at 14500 rpm for 30 min in room temperature. Supernatant was transferred in new tube and added 1x volume of chloroform, shaken for 15 minutes, and centrifuged at 14000 rpm for 10 minutes. The supernatant was transferred in new tube, added with Na-acetate 3 M 0.1x volume with different pH, (i.e pH 8.5 for porcine, pH 7.6 for bovine, and both pH for series concentration of capshells considering proportion of porcine and bovine gelatine), added with cold ethanol absolute 2x volume, and incubated in -80°C overnight. Subsequently, it was centrifuged at 14500 rpm for 5 min at 4°C. Immediately, the supernatant is cleaned from any residual ethanol for about 10 minute in laminar air flow (LAF). Finally, the mixture was added with 30-50 µL TE buffer. The DNA pellet was stored at -20°C for further analysis.

PCR amplification

DNA amplification using D-loop primers was performed in 20 µL final volume, consisting of 10 µl evagreen® master mix, 0.5 µL forward primer and 0.5 µL reverse primer (each concentration is 10 µM), 1 µL of DNA template (50 ng), and nuclease free water. Amplification assay was set on initial denaturation at 95°C for 1 min, denaturation at 95°C for 20 sec, annealing at optimum temperature for 30 sec, and elongation at 72°C for 45 sec.

Determination of sensitivity and repeatability of the assay

Sensitivity assay of D-loop 575 and D-loop 93 primers was expressed as detection limit of bovine DNA in pure gelatine and capshells. The replicate of real-time PCR measurements was made by dilution series of bovine gelatin (1000, 500, 200, 150, 100, 10, 5, and 1 pg µL^{-1}). The limit of detection (LoD) was determined as the lowest amount of DNA that could be amplified with reproducible Cq value.

Results and Discussion

In this study, bovine gelatine DNA was identified using RT-PCR using new specific primer for D-loop sequence of bovine. The primers used had short amplicon, less than 250 bp in order to increase the efficiency of PCR method. In addition, GC content of primers is in the optimum range of 40-60%, since GC content will affect stickiness of 3' end (Muhammed *et al.*, 2015). DNA extraction was performed by phenol-Choloroform-isoamyl alcohol method according to Sambrook *et al.* (1989). Preparation step involved the use of phosphate buffer saline (PBS) containing various salts. The process of extraction DNA occurs through several steps, namely destruction of cell membranes (lysis), degradation RNA using RNAse, separation protein and contaminants to draw DNA using organic solvents (extraction DNA), purification, precipitation and concentration. The concentration and purity of isolated DNA from pure gelatine and capshells were measured using spectrophotometer UV at λ 260 and 280 nm, respectively. The purity of DNA obtained is in range 1.04–1.86, and DNA concentration is in the range of 120–435 µg mL^{-1}.

The designed primers were optimized toward several DNAs extracted from fresh animal tissues (pig, cows, chickens, goats, rats, and wild boar) in order to determine the optimum annealing temperature at range of 48–56°C. The number of cycles is limited to 35. Primer D-loop 575 showed DNA amplification of wild boar, goats, rats, and cows, whilst primer D-loop 93 showed amplification of cows DNA at 48.4°C, 50°C, 51.4°C and 52.2°C, and low amplification of goat DNA at 48.4°C (Figure 1). This result was confirmed by running all DNA during 30 cycles of amplification at 51.4°C, since it was the optimum temperature which had the highest relative fluorescence unit (RFU) value. The cow's DNA is amplified with low number of cycles, has one peak with high RFU value, therefore primer D-loop 93 was chosen for further analysis (Figure 2). Primer D-loop 93 was subjected to specificity test toward porcine and bovine DNA from gelatine, and it showed specific amplificaiton on bovine DNA (either from fresh tissue animal or bovine gelatine). Cycle number of bovine DNA is approach to that of cow DNA with one peak and high RFU value (Figure 3). Amplification was also performed on prepared capshells.

The sensitivity of RT-PCR using D-loop 93

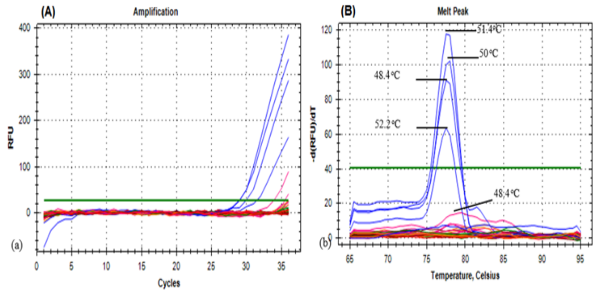

Figure 1. (A) Amplification curve of DNA from fresh tissue animals using primer D-loop 93 at different annealing temperatures and (B) Melting curve analysis of during the amplification. Blue : cows DNA, pink : goats DNA, the other colour are others DNA tissue animals using primer D-loop 93 at different annealing temperature.

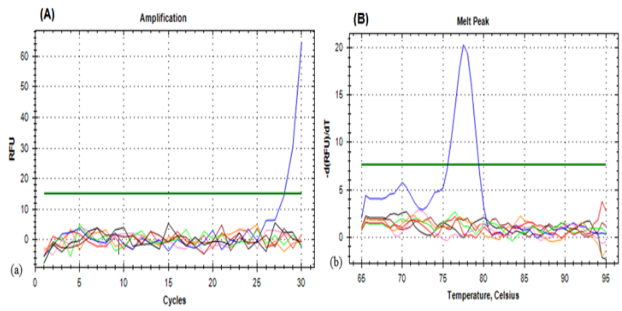

Figure 2. Confirmation of specificity test primer D-loop 93 at 51.4°C (a) Amplification curve of DNA from fresh tissue animals using primer D-loop 93 and (b) Melting curve analysis of during the amplification. Blue : cows DNA, pink : goats DNA, the other colour are others DNA.

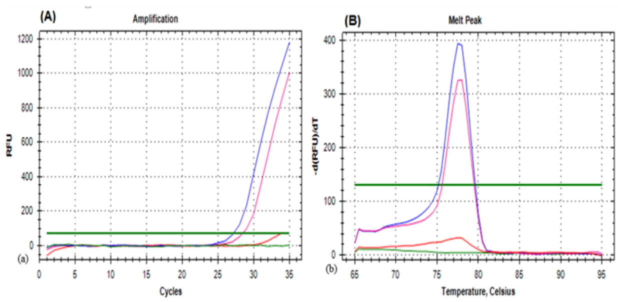

Figure 3. (A) Specificity test of primer D-loop 93 at 51.4°C toward bovine gelatine, porcine gelatine, and cows DNA (a) Amplification curve of DNA showed only the amplification of bovine gelatine and cows DNA and (b) Melting curve analysis of during the amplification. Blue : cows DNA, pink : bovine gelatine DNA, red : porcine gelatine DNA, green : No Template Control (NTC).

was expressed by limit of detection (LoD). For determining LoD, dilution series of bovine gelatine DNA (1000, 500, 200, 150, 100, 10, 5, and 1 pg) are used. Bovine DNA can still be amplified up to 5 pg, while at 1 pg there was no amplification. Therefore, it can be judged that LoD value of DNA to be amplified is 5 pg. The R^2 obtained for the relationship between log of DNA concentration (axis) and cycle threshold

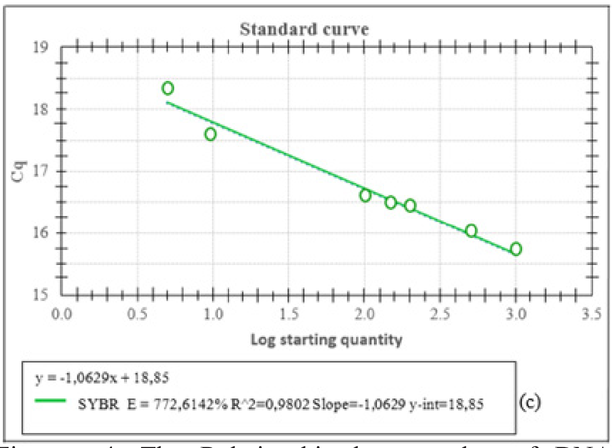

Figure 4. The Relationship between log of DNA concentration and cycle treshold (Cq)

(Cq) was 0.9802, with y-intercept of 18.85, and amplification efficiency (E) is 772.6142% (Figure 4). The ideal E value is 90-110% as recommended by Codex Allimentareus Commission (CAC, 2010).

The value of E was affected by some factors, such as assay performance, which depend on primer and template sequences and structure, inhibitors and other interfering substances from sample or carry overs agents from upstream processing steps, grade of reagents and its concentrations, and the presence of competing reactions (Svec *et al.*, 2015). The criteria of qualitative and quantitative real-time PCR method are to comply $R^2 \geq 0.98$ and E of 90–110% (Broeders *et al*., 2014). The high E value obtained can be caused by inhibitors present in the mixture with high concentration or inconsistent in pipeting small volume, which caused poor precision (Muhammed *et al*., 2015). Standard curve was also prepared from bovine-porcine gelatine in prepared capshells (0, 50, 60, 70, 80, 90, and 100% w/w). The R^2 obtained is 0.9681 with E = 192.8141%.

Repeatability test was performed at two serial dilutions in standard curve (100 and 10 pg) with three repetitions. The coefficient of variation (CV) obtained is 1.03%, which is 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 capshells 100% bovine gelatine. The CV value of 1.35% was obtained. The primer D-loop 93 was also performed toward bovine gelatine DNA in commercial capshells samples. All samples were amplified, meaning that commercial capshells samples contain bovine gelatin DNA (Figure 5).

Conclusion

Primer D-loop 93 with 64 bp amplicons length can specifically identify the presence of bovine

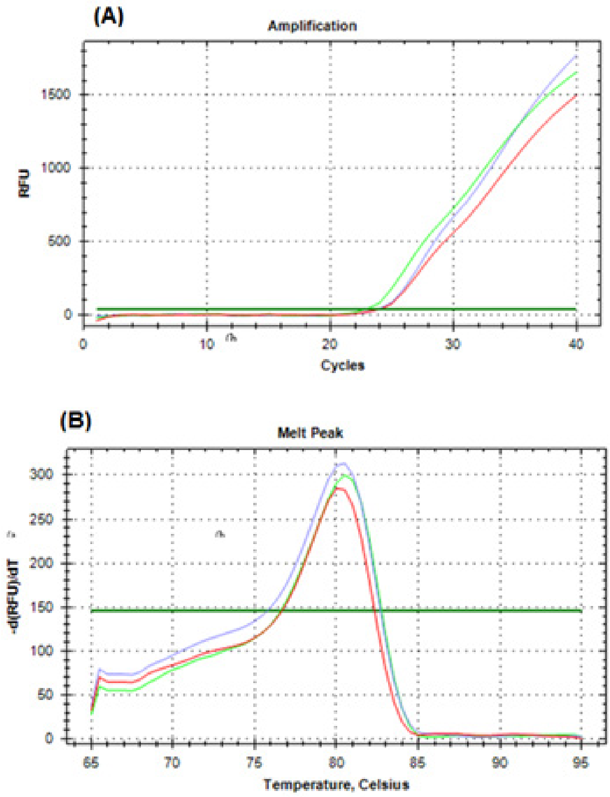

Figure 5. The results of detection bovine DNA in commercial capshells. (A) Amplification curve of extracted DNA from commercial capshells and (B) Melting curve analysis during the amplification.

DNA in fresh tissue and gelatine sources at optimum annelaing temperature of 51.4°C. The limit detection of bovine DNA was 5 pg. The coefficient of variation (CV) on repeatability analysis was 1.03%.

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