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Primer rRNA-12S for detection of bovine gelatine DNA in capsule shells using real-time polymerase chain reaction

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Abstract

About 90% gelatine used in food and pharmaceuticals comes from porcine gelatine. The presence of porcine gelatine is hindered by Muslim society; therefore the confirmation of halal gelatine like bovine gelatine is a must. In pharmaceuticals, gelatine is used for making capsule shells. Analysis of the capsule shells containing bovine gelatine via DNA analysis is required in order to verify the halalness status of gelatine sources. The objective of this study was to develop real-time polymerase chain reaction (real-time PCR) for identification of bovine gelatine in capsule shells. In this study, a couple of bovine-specific primer, rRNA-12S primers, (forward: 5'-CCC AAG CTA ACA GGA GTA CG-3' and reverse: 5'-TAG TGC GTC GGC TAT TGT AG-3') were designed. The annealing temperature of primer was optimized to get optimum amplification of DNA target at 51.1-58.7°C. The primer was subjected to specificity test using DNAs isolated from fresh chicken, pork, wild boar, lamb, and beef as well as commercial porcine gelatine and bovine gelatine and capsule shells made from the mixture of porcine and bovine gelatines. The used primer successfully amplified bovine gelatine DNA. Primers also showed high specify toward capsule shells made from the mixture of porcine-bovine gelatines. The sensitivity test resulted primers enable to amplify up to 0.48 ng of bovine gelatine DNA, and 1.2 ng of DNA extracted from bovine gelatine capsule. The rRNA-12S primers were finally applied to identify bovine DNA in commercial gelatine capsules.

Introduction

Capsule is one of the popular dosage forms due to its advantages, including attractive appearance, ease of use, portability, convenience administration, and flavourless. Capsule, either soft or hard capsule shells, is mostly made from gelatine (Sudjadi *et al*., 2016). Gelatine is a protein derived from collagen. Gelatine has solubility, viscosity and thermally reversible gelatinous properties that meet technical needs for capsule raw materials. Gelatine-based capsules have following characteristics: strong, clear, flexible, and high-gloss films. Besides, they dissolve readily under the existing conditions in the stomach. Gelatines are obtained from pig skin, beef hide, and bones of cow and pig (Schrieber and Gareis, 2007). The presence of porcine and bovine gelatines has some concerns due to the halalness status. The porcine gelatine is not allowed to be used in pharmaceuticals by Muslim society unless in the extreme conditions. As a consequence, the bovine gelatine must be used because it is halal according Syariah law and fits for Muslim society (Mursyidi, 2013).

Bovine and porcine gelatines have similar physico-chemical properties and are difficult to be distinguished, particularly in the processed foodstuffs (Widyaninggar *et al*., 2012). Some analytical methods have been reported for differentiation and classification of gelatine sources, mainly porcine and bovine gelatines, namely Fourier transform infrared spectroscopy (Hashim *et al*., 2010; Nur Cebi *et al*., 2016), high performance liquid chromatography (HPLC) using fluorescence detector via analysis of amino acid composition (Nemati *et al*., 2004), HPLC-mass spectrometry through analysis of peptides (Flaudrops *et al*., 2015; Grundy *et al*., 2016), and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) for distinction polypeptide patterns of gelatines (Nur Azira *et al*., 2014). These methods are not specific enough and are difficult to differentiate gelatines in the complex matrices like food and pharmaceuticals. Fortunately, polymerase chain reaction (PCR) for DNA analysis offers specific and sensitive detection for certain DNA species, including bovine DNA extracted from gelatine materials.

PCR technology offers sensitive and specific method to identify certain species in processed foodstuffs using species specific primers (Bellagamba *et al*., 2001). PCR method enable to amplify DNA

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fragment and produce up to millions copies, even when the sample is limited and has undergone degradation (Elrich, 1989; Fumiere *et al.*, 2006). The introduction of real-time PCR has revolutionized its uses in any aspects, including halal authentication in food and pharmaceutical products (Rohman *et al*., 2016). PCR along with its development has been used for analysis of nonhalal meat for authentication studies. PCR using restriction fragment length polymorphism (PCR-RFLP) with cytochrome b gene as target has been used for the identification of pork in meatball products (Erwanto *et al.*, 2014) and for identification of pork in the beef sausage and chicken nugget, including before and after frying (Erwanto *et al.*, 2011). Guntarty *et al.* (2017) and Widyasari *et al*. (2015) have used real-time PCR for analysis of DNAs extracted from wild boar meat and rat's meat, respectively, using species specific primers.

In the analysis of gelatine sources, our group have developed real-time PCR for analysis of porcine gelatine in capsule shells and in food products using primers D-loop region of mitochondrial DNA (mtDNA) (Sudjadi *et al*., 2016; Sepminarti *et al*., 2016). However, it is very limited related to the use of real-time PCR methods intended to analyse bovine gelatin. In this study, analysis of bovine DNA in gelatine capsule was performed using species specific primer targeted to amplify mtDNA particularly rRNA-12S sequence.

Materials and Methods

Primer design was facilitated by Primer-BLAST provided in NCBI website. The target sequence is *Bos Taurus* mtDNA with number of accession AF 492351. The selected primers were then ordered and were obtained from IDT (Singapore) and used for real-time PCR analysis.

Sample preparation

A total of 3 levelled matrix samples were prepared. Firstly, fresh tissues of chicken, pork, wild boar meat, lamb, and beef. Secondly, analytical grades of bovine gelatine (Type B) and porcine gelatine (Type A) purchased from Sigma-Aldrich (St. Louis, MO, USA). Thirdly, reference capsule shells and commercial gelatine capsules. Reference capsule shell was a self-made capsule shell and was prepared according to Widyaninggar *et al.* (2012). A total of 3 gram of binary mixtures of bovine-porcine gelatine with concentration varies from 5, 10, 20, 30, 75, and 100% wt/wt bovine gelatine was prepared as reference capsule shells. The samples of commercial gelatine capsules were obtained from 3 different manufacturers and were labelled as O, C, and B.

DNA extraction

DNA extractions from fresh tissues were done using DNeasy Blood & Tissue Kit according to procedure described by manufacturer's protocol. The addition of RNase (Sigma, USA) was done to prevent RNA contamination. Meanwhile, DNA extraction of gelatines and capsule shells were conducted according to Sambrook *et al.* (1989). Subsequently, quality and quantity of extracted DNA were determined by absorbance measurement in λ 260nm and λ 280nm using spectrophotometer UV.

Real-time PCR analysis

Real-time PCR was carried out using CFX96 Real-time PCR instrument (Bio-rad, USA). A total of 20 µL PCR reagent consisting of Ssofast EvaGreen super mix, a couple of primers at final concentration of 500 nm each, template DNA, and nuclease-free water was prepared. Real-time PCR is programmed for short protocol as follows: initial denaturation (95°C, 30 seconds), denaturation (95°C, 5 seconds), annealing (55.8°C, 10 seconds), extension (72°C, 10 seconds), and final extension (72°C, 5 minutes). The melt curve protocol was performed as instrument default setting. An amount of 30 ng bovine DNA was amplified in 5 different annealing temperatures in the range of 51.1-58.7°C to determine optimum condition. The optimization was also performed for determining optimum amount of gelatine DNA (50, 75, 100, and 150 ng) used in PCR amplification.

Validation of real-time PCR analysis

To perform validation of real-time PCR analysis, each validation parameter was evaluated using Codex Alimentarus Comission (2010). The specificity of primer was examined toward DNA extracted from 5 kinds of fresh tissues (30 ng of each DNA), porcine and bovine gelatine, as well as reference capsule shells. The optimum condition and primer specificity were determined according to evaluation of amplification plot and melt peak. The limit of detection (LoD) of DNA extracted from bovine gelatine and bovine gelatine-made capsules was determined through the amplification of total 5 concentration series. Repeatability test was observed using three replicates of amplification reaction for each sample.

Results and Discussion

In this study, bovine mtDNA (accession no. AF 492351) was used as amplification target with

Table 1. Characteristics of rRNA-12S primers used for detection of bovine gelatine DNA.

Primer sequence		Product size	Gene	Melt temp. (°C)	Accession number
Forward	5'-CCC AAG CTA ACA GGA GTA CG-3'	137 bp	rRNA-12S	57.70	AF 492351
Reverse	5'-TAG TGC GTC GGC TAT TGT AG-3'			57.23	

amplicon size range of 100-200 base pairs (bp). MtDNA was found to provide multiplied number of DNA copies as PCR amplication target, and was expected to give better sensitivity and overcome issues of minute quantity of the extracted DNA. This issue was mainly found when sample has undergo degradation such as gelatine and capsule made from gelatine which resulted the fragmentation of contained DNA (Mohamad *et al*., 2016). The sequence of DNA target was obtained from NCBI gene database. Through Primer-BLAST, 10 pairs of primer candidates displayed and surprisingly all of them amplified a fragment in rRNA-12S region which was part of *Bos Taurus* mtDNA (data not shown). This has confirmed the specificity of rRNA-12S coding region upon other regions in mtDNA sequence. Moreover, rRNA-12S region have been used for amplification target in numbers of species-specific identification previously (Mafra *et al*., 2007; Kumar *et al.*, 2012). Finally, only a pair of primer was selected which was specific toward mtDNA according to Primer-BLAST evaluation (Table 1). The primer was called rRNA-12 primers and was known to amplify rRNA-12S region covering base sequence 332 tp 468 (AF 492351).

Looking for the optimum condition in PCR reaction is important aspect to be considered prior to real-time PCR analysis. In this study, the optimization of annealing temperature and gelatine DNA amount were performed. The results showed that optimum amplication was observed at annealing temperature of 55.8°C. This annealing temperature was furthermore used in every real-time PCR analysis. Meanwhile, the optimum amount of gelatine DNA was 75ng which produced best value of highest amplification curve. Beside those optimizations, the adjustment of real-time PCR duration was also considered. Generally in PCR, each step spends 30 up to 60 seconds. Nevertheless, recent study by Mao *et al*. (2007) revealed that EvaGreen is suitable for fast cycling protocol without adversely affecting real-time PCR performance. For this reason, in this analysis, a fast protocol at 5-10 seconds per step was introduced

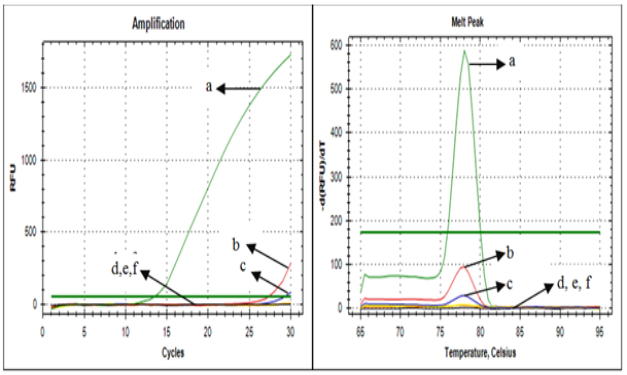

Figure 1. The amplification plot (A) and melt peak (B) of rRNA-12S primers toward five species DNAs at 30 ng. DNA extracted from (a) beef; (b) lamb; (c) chicken; (d) pork; (e) wild boar meat; (f) no template control. Y-axis represents Relative Fluorescent Unit (RFU). X-axis = the number of cycles.

which beneficially cut the analysis duration.

The specificity test confirmation showed that rRNA-12S primers were not fully specific toward bovine DNA, as lamb DNA and chicken DNA were also amplified at last cycle (Figure 1A). However, both DNA of lamb and chicken were amplified at last cycles with quantitation cycle (Cq) of 27.21 and 29.15 respectively, compared to bovine DNA (Cq = 13.37), therefore, one can adjust the number of cycles is limited to 25 in order to assure specificity of bovine DNA. Lower rate of Cq showed that primer rRNA-12S provide better specify. Furthermore, the used primers successfully amplified bovine gelatine DNA with Cq of 25.6, while porcine gelatine DNA was not amplified by rRNA-12S primers until 35 cycles.

The extensive degradation during acidic and basic hydrolysis involved in manufacture of gelatine resulted the low content DNA in the sample. To overcome this problem, the amount of starting material is increased to 500 mg. Poor quality of DNA extracted from gelatine is considered by strong interaction between extracted DNA and non-lysed gelatine residues (Mohamad *et al.,* 2016). In order to enhance quality and prevent contamination, some attempts were employed during DNA extraction, namely omitting vortexing lysis step, increasing amount of proteinase K and repeating phenol extraction to minimize protein contamination, and preventing RNA contaminant by addition of RNase.

Figure 2(A) was standard curve for determination of limit of detection (LoD) of DNA extracted from bovine gelatine. LoD value reported was 0.48 ng with cycle quantification (Cq) value of 36.84. The satisfaction results on linearity and efficiency were observed on standard curve of bovine gelatine DNA, in which linearity was shown by r^2 value of 0.981 (> 0.980) and efficiency at 108.2% (90-110%). Both

Table 2. The repeatability test of quantitation cycle (Cq) expressed by coefficient variation (CV) using different DNAs extracted from different samples.

Sample	DNA quantity (pg)	Cq	Cq mean	SD	CV (%)
Bovine gelatine	6000	34.17 33.90 33.64	33.90	0.26	0.78
Capsule shells containing 30% bovine gelatine	75000	33.79 34.17 34.00	33.98	0.19	0.56
Bovine gelatine capsule shells	12000	35.05 34.63 34.66	34.78	0.23	0.67

values of r^2 and E fulfilled the method validation criteria as guided by Codex Alimentarus Comission (2010). The efficiency (E) value reflected how close the amplicon product in the experimental to that of theoretical state (100%) according to doubling theory (Pelt-Verkuil *et al*., 2008). The efficiency value (E) greater than 100% may be caused by pipetting error or non-specific product amplification (Bio-Rad, 2006). The lower E value is a common issue in real-time PCR amplification using intercalating dye (Smith and Osborn, 2009), therefore it is very important to perform further studies involving specific-sequence probe to give better understanding on actual efficiency and confirm the occurrences of non-specific amplification. The specificity test was also conducted toward DNA extracted from bovine gelatine capsules. Primer rRNA-12S was able to amplify DNA extracted from bovine gelatine capsule up to 1.2 ng at Cq value of 36.32. The standard curve was shown in Figure 2(B), with r^2 and E values were of 0.976 and 122.8%, respectively. The results of repeatability test, expressed by coefficient of variation (CV), was compiled in Table 2. The acceptable CV value was < 25% according to Codex Alimentarus Comission (2010). The CV values reported was in the range of 0.56-0.78%, which fulfilled the criteria of repeatability test (Table 2).

The real-time PCR using rRNA-12S primers were furthermore used for analysis of bovine gelatine in the commercial capsule shell. Figure 3 showed the amplification curve of DNA extracted from commercial capsule shells. From three samples, only one capsule (C) was positively amplified by primer rRNA-12S, similar to curve profile in the positive control. Meanwhile, two other commercial capsules were not amplified until 40 cycles. Nevertheless,

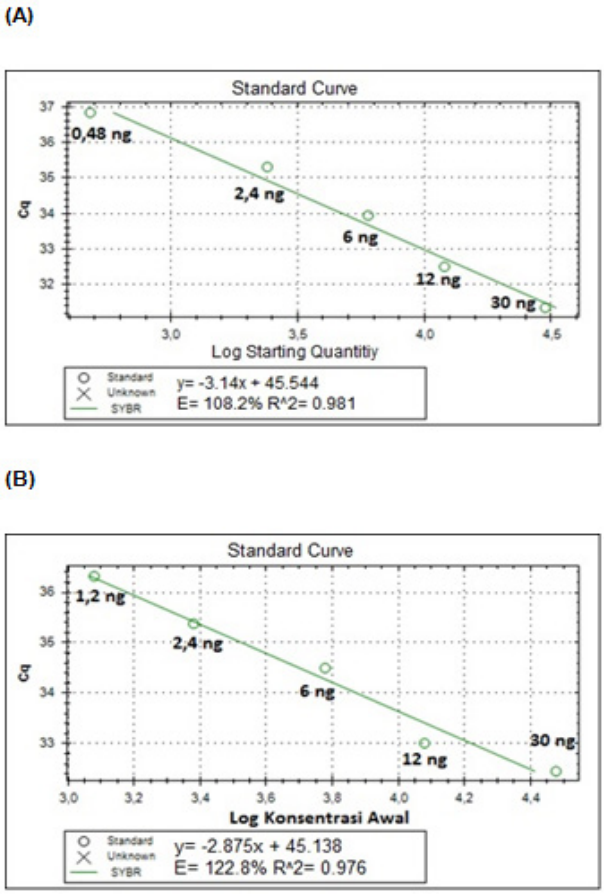

Figure 2. Standard curve for determining efficiency and limit of detection. (A) DNA extracted from commercial gelatine; (B) DNA extracted from capsule shell made from bovine gelatine. X-axis represents amount of DNA (pg) in logarithmic function and Y-axis represents the quantitation cycle.

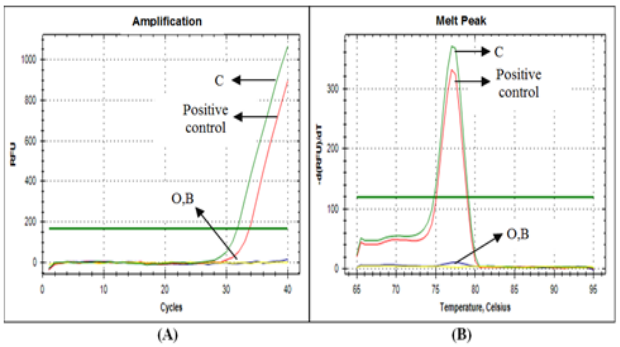

Figure 3. Amplification plot (A) and melt peak (B) of DNA extracted from commercial capsules using rRNA-12S primers.

further investigation might be required toward these two commercial capsules (O and B). In order to confirm the species origin, it is suggested to identify DNA types which are specific to porcine gelatine, considering that bovine and porcine gelatines are gelatine types mostly used in capsule shells.

Conclusion

The rRNA-12S primers was suitable for analysis of bovine gelatine DNA extracted from commercial gelatine and that extracted from the capsule shell containing bovine gelatine for halal verification. Furthermore, the applicability of short protocol for real-time PCR involving EvaGreen dye was confirmed, thus shorter duration of analysis could be achieved by this method using rRNA-12S primers.

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