



## Analytical Methods

## Halal authenticity of gelatin using species-specific PCR



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## ABSTRACT

Consumption of food products derived from porcine sources is strictly prohibited in Islam. Gelatin, mostly derived from bovine and porcine sources, has many applications in the food and pharmaceutical industries. To ensure that food products comply with halal regulations, development of valid and reliable analytical methods is very much required. In this study, a species-specific polymerase chain reaction (PCR) assay using conserved regions of mitochondrial DNA (cytochrome b gene) was performed to evaluate the halal authenticity of gelatin. After isolation of DNA from gelatin powders with known origin, conventional PCR using species-specific primers was carried out on the extracted DNA. The amplified expected PCR products of 212 and 271 bp were observed for porcine and bovine gelatin, respectively. The sensitivity of the method was tested on binary gelatin mixtures containing 0.1%, 1%, 10%, and 100% (w/w) of porcine gelatin within bovine gelatin and vice versa. Although most of the DNA is degraded due to the severe processing steps of gelatin production, the minimum level of 0.1% w/w of both porcine and bovine gelatin was detected. Moreover, eight food products labeled as containing bovine gelatin and eight capsule shells were subjected to PCR examination. The results showed that all samples contained bovine gelatin, and the absence of porcine gelatin was verified. This method of species authenticity is very useful to verify whether gelatin and gelatin-containing food products are derived from halal ingredients.

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## 1. Introduction

At present, gelatin is a very popular ingredient in various food and pharmaceutical products. Gelatin is, by far, most widely used in confectionery products and pharmaceutical capsules around the world (Schrieber & Gareis, 2007). Gelatin is a mixture of polypeptides obtained by the partial hydrolysis of collagen (Cheng et al., 2012). In the large-scale manufacture of gelatin, the primary raw material used is the collagen found in cattle and pig (Schrieber & Gareis, 2007). Commercial forms of gelatin such as sheets, granules, or powders are mainly sourced from bovine bone and hides, pig skin, and, more recently, pig bone (Hermanto & Fatimah, 2013; Jaswir, Mirghani, Mohd Salleh, Hassan, & Yaakob, 2009). Other sources such as fish and poultry are very new and are mostly processed to cater to specific religious consumer groups (Gómez-Estaca, Montero, Fernández-Martín, & Gómez-Guillén, 2009; Karim & Bhat, 2009).

In Islam, consumption of products considered non-halal, such as those containing porcine gelatin, is strictly forbidden (Widyaninggar, Triyana, & Rohman, 2012). Some Islamic countries have established strict regulations for producers and importers to stamp their products with a halal certificate to distinguish them from non-halal products (Hermanto & Fatimah, 2013). In this regard, development of reliable and sensitive analytical methods for evaluating halal authenticity is very critical. Methods that rely on protein analysis or physicochemical properties, such as chemical precipitation (Hidaka & Liu, 2003), Fourier transform infrared spectroscopy (Hashim et al., 2010), electrophoretic analysis (Hermanto & Fatimah, 2013), high-performance liquid chromatography (Nemati, Oveisi, Abdollahi, & Sabzevari, 2004), mass-spectrometer detection (Zhang et al., 2009), and enzyme-linked immunosorbent assay (Venien & Leveux, 2005), have been applied to differentiate bovine gelatin from porcine gelatin. These methods proved to be unsuitable for differentiating a mixture of gelatin as well identifying the origin of gelatin in food products. This is mainly because of the similarities in structure and physicochemical properties of gelatin derived from different sources. Moreover, the presence of highly degraded proteins due to extreme

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**Table 1**

The characteristics of bovine and porcine primers.

| Species | Primer sequences 5'–3'                                      | Product size | Genes | Ann. temp. (°C) | Accession number |
|---------|-------------------------------------------------------------|--------------|-------|-----------------|------------------|
| Bovine  | 5'-GCCATATACTCTCCTTGGAACA-3'<br>5'-GTAGGCTTGGGAATAGTACGA-3' | 271 bp       | Cyt b | 58              | J01394           |
| Porcine | 5'-GCCTAAATCTCCCTCAATGGTA-3'<br>5'-ATGAAAGAGCAATAGATTTCG-3' | 212 bp       | Cyt b | 58              | AF039170         |

temperature and pH treatments during gelatin production render these methods almost unreliable (Tasara, Schumacher, & Stephan, 2005).

Polymerase chain reaction (PCR) can be used as an alternative method for rapid and sensitive detection of porcine DNA in gelatin due to the higher stability of DNA compared to protein (Aida, Man, Yaakob, Raha, & Son, 2007). Earlier reports have also described PCR using species-specific primers in highly processed food products (Calvo, Zaragoza, & Osta, 2001; Ghovvati, Nassiri, Mirhoseini, Moussavi, & Javadmanesh, 2009; Hidaka & Liu, 2003; Jia-qin et al., 2008; Murugaiah, Mustakim, Mohd Noor, & Radu, 2010). There are a limited number of reports available on animal species-specific identification of gelatin, gelatin-containing food, and pharmaceutical products using PCR techniques (Cai, Gu, Scanlan, Ramatlapeng, & Lively, 2012; Demirhan, Ulca, & Senyuva, 2012; Tasara et al., 2005). Real-time PCR is sensitive and specific enough to trace small amounts of target DNA; hence, it is successfully applied in the detection of bovine and porcine DNA in gelatin mixtures, gelatin-containing food products, and capsule shells. However, due to the high cost of real-time equipment and reagents, not all laboratories are able to apply this method. Conventional PCR using species-specific PCR has been shown to be sensitive in the detection of 0.1% of bovine gelatin in porcine gelatin, but it has not been employed in terms of identifying the animal origin of the gelatin used in food and pharmaceutical products.

The present study aimed to perform conventional PCR using species-specific primers for identifying the animal origin of gelatin powders and to investigate the halal authenticity of some gelatin-containing food products and capsule shells.

## 2. Material and methods

### 2.1. Sample preparation

Gelatin samples of known species origin, bovine and porcine, were received in powder form from the Food and Drug Organization of Iran and used as reference standards. Binary gelatin mixtures were prepared by adding 0.1%, 1%, 10%, and 100% (w/w) of porcine gelatin into bovine gelatin and vice versa. A total of 16 samples were prepared for the present study. Eight pharmaceutical capsule shells of unknown species origin were collected from pharmacies in Tehran. Food products labeled as containing bovine gelatin including two marshmallows (imported from Turkey), two jellies, two desserts, and two cakes were purchased from supermarkets in Tehran.

### 2.2. DNA extraction

DNA was extracted from pure gelatin powders, capsule shells, and gelatin-containing food products, using a DNeasy mericon Food Kit (Qiagen, Hilden, Germany), Small Fragment Protocol (2 g), recommended for highly processed foods. Initially, 10 ml of lysis buffer and 25 µl of proteinase K (20 mg) solution were added to 2 g of the sample, vortexed briefly, and incubated for 30 min at 60 °C in a water bath (Mettler, Schwabach, Germany) with

constant shaking. After incubation, the solution was cooled to room temperature (15–25 °C) on ice and then centrifuged (Eppendorf, Hamburg, Germany) for 5 min at 2500×g. Subsequently, 700 µl of the clear supernatant was transferred to a microcentrifuge tube containing 500 µl of chloroform, vortexed vigorously for 15 s, and centrifuged at 14,000×g for 15 min. After centrifugation, 250 µl of the upper aqueous phase was added into a fresh microcentrifuge tube containing 1 ml of binding buffer and mixed thoroughly by vortexing. Then, 600 µl of the mixture was pipetted into a spin column placed in a collection tube, centrifuged at 17,900×g for 1 min, and the flow-through was discarded. This step was repeated with the remaining sample. Afterwards, 500 µl of the wash buffer was added to the spin column, followed by centrifugation at 17,900×g for 1 min, and the flow-through was discarded. The collection tube was centrifuged again at 17,900×g for 1 min to dry the membrane. Finally, the spin column was transferred to a 1.5-ml fresh microcentrifuge tube, and 30 µl of elution buffer was added onto the membrane, incubated for 1 min at room temperature (15–25 °C), and then centrifuged at 17,900×g for 1 min to elute. The purified DNA solution (30 µl) was immediately used.

### 2.3. Species-specific primers and PCR amplification

The information about two sets of primers for bovine and porcine used for PCR amplification is listed in Table 1. These primers were published by Tartaglia et al. (1998) and Lahiff et al. (2001) for bovine and porcine DNA, respectively. PCR amplification was carried out using a master thermal cycler (Eppendorf, Germany). The 20-µl reaction mixture was prepared in a PCR tube with 2 µl of 10 × PCR buffer (CinnaGen Co, Tehran, Iran), 0.2 mM of deoxyribonucleoside triphosphates (dNTPs) (CinnaGen Co, Iran), 1.5 mM MgCl<sub>2</sub> (CinnaGen Co, Iran), 0.05 units of Taq DNA polymerase (CinnaGen Co, Iran), 0.5 µM each of forward and reverse primers listed in Table 1 (Eurofins MWG Operon, Germany), 10–100 ng of DNA template, and nuclease-free water to adjust the volume (CinnaGen Co, Iran).

The PCR program used for amplification of the target genes is as follows: initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 40 s, and extension at 72 °C for 30 s. Then final extension was conducted at 72 °C for 5 min. The amplified products of PCR were analyzed using 2% agarose gel in TBE (Tris–borate–ethylenediaminetetraacetic acid) 0.5× buffer stained with DNA safe stain (CinnaGen Co, Iran) as a visualizing agent and run for 45 min at 100 V.

## 3. Results and discussion

In highly processed food products such as gelatin and gelatin-containing food and pharmaceutical products, DNA is degraded into short fragments. This issue caused some difficulties in PCR amplification as reported by earlier researchers (Mafra, Ferreira, & Oliveira, 2008; Martín et al., 2007). The essential prerequisite for PCR amplification is therefore obtaining sufficient template DNA for analysis. For this reason, DNA isolation was performed using a commercial kit under optimized column-binding conditions adjusted for maximal recovery of short DNA fragments.

Many primers have been published based on both mitochondrial and nuclear genes to trace either bovine or porcine DNA in a variety of food products. In this experiment, to detect the presence of small amounts of DNA in gelatin, primers were selected from conserved regions of mitochondrial genes (cytochrome b). The high copy number of mitochondrial DNA per cells and the probability of their survival under different processing conditions ensure amplification of the expected PCR products even in samples containing small amounts of DNA (Rodriguez et al., 2004). Furthermore, primer-binding sites were selected to amplify specific short fragments of DNA, because of degradation due to gelatin processing.

In the first step, primers were applied to amplify extracted DNA from gelatin powders with known origin. The gel electrophoresis results for the PCR amplified products revealed expected bands of 212 and 271 bp for porcine and bovine gelatin, respectively. The specificity of the method was tested using DNA obtained from gelatin powders of bovine and porcine origin, against animal meat species including donkey, sheep, horse, chicken, turkey, and goat, and no cross contamination was observed (Fig. 1). Tasara et al. (2005) employed a selection of one bovine and four porcine primer sets, using conventional PCR, and they showed that the bovine primer pair was able to detect bovine DNA in gelatin templates specifically. However, among the four porcine primer sets, only one primer pair detected target DNA in the gelatin template, but it was nonspecific and cross-reacted with the bovine DNA template.

In the second step, the detection limit of the method was tested on different handmade admixtures containing 0.1%, 1%, 10%, and 100% (w/w) of porcine gelatin within bovine gelatin and vice versa. The PCR was performed using porcine primers (Fig. 2) and bovine primers (Fig. 3). As shown, porcine and bovine gelatin content as low as 0.1% was detected. These results clearly showed that conventional PCR is sensitive enough for detecting considerably low

percentages of bovine and porcine gelatin. Therefore, the high sensitivity of this assay is suitable to ensure that food and pharmaceutical products comply with religious convictions.

Finally, the assay was employed for the detection of bovine and porcine DNA in some gelatin-containing food products and capsule shells to certify their halal authenticity. The results showed that all products including eight capsule shells, two marshmallows, two cakes containing gelatin, two jellies, and two desserts contained bovine gelatin. PCR analysis for detecting porcine DNA also showed all samples to be negative. As an example, the results of gel electrophoresis for DNA templates isolated from six samples tested with both porcine and bovine primer pairs are shown in Fig. 4. Except for marshmallows, which were imported from Turkey, the other products were manufactured in Iran in compliance with food regulations. These findings showed that the tested food and pharmaceutical products were correctly labeled as halal.

DNA fragments >200 bp in length were amplified with the method described in this study, which indicates that degradation of DNA cannot present a major obstacle for performing PCR techniques. The findings of this study indicated that difficulties in

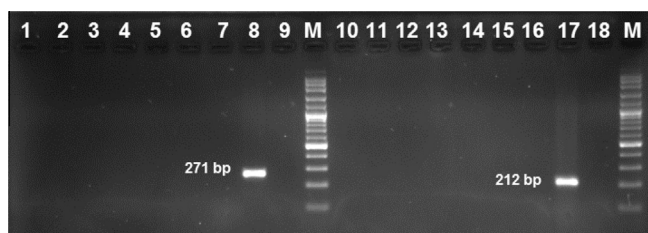


**Fig. 1.** PCR specificity for bovine- and porcine-specific primers. Lanes 1–8 are donkey, goat, chicken, horse, pig, turkey, sheep, and cattle DNA, respectively, tested with bovine primers; lanes 10–17 are donkey, goat, chicken, horse, cattle, turkey, sheep, and pig DNA, respectively, tested with porcine primers. Lanes 9 and 18 are negative controls. M, DNA marker (100 bp).

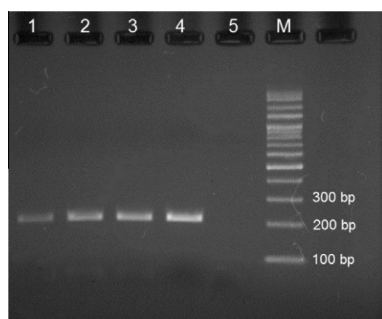


**Fig. 2.** Agarose gel electrophoresis of PCR product amplified from different mixtures of porcine gelatin with bovine gelatin using porcine primers; lanes 1–4 show porcine DNA of 0.1%, 1%, 10%, and 100%, respectively; lane 5, negative control; and M, DNA marker (100 bp).

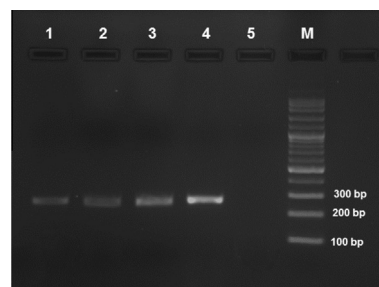


**Fig. 3.** Agarose gel electrophoresis of PCR products amplified from different mixtures of bovine gelatin within porcine gelatin using bovine primers; lanes 1–4 show bovine DNA of 0.1%, 1%, 10%, and 100%, respectively; lane 5, negative control; and M, DNA marker (100 bp).

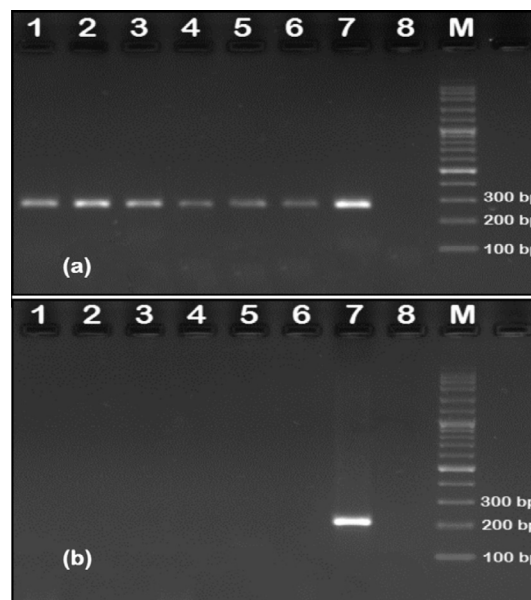


**Fig. 4.** Agarose gel electrophoresis for PCR products amplified from isolated DNA of different food samples and two capsule shells tested with bovine (a) and porcine (b) primer pairs: lanes 1 and 2, capsule shells; lane 3, jelly; lane 4, cake containing gelatin; lane 5, dessert; lane 6, marshmallow; lane 7, positive control; lane 8, negative control; and M, DNA Marker (100 bp).

PCR amplification of short DNA fragments can be overcome using high-efficiency primers and suitable extraction methods.

#### 4. Conclusion

A conventional PCR technique using species-specific primers was performed for identifying the animal origin of gelatin powders and gelatin used in the manufacture of food products and capsule shells. The results of this study showed the applicability of the method for identifying bovine and porcine gelatin in food and pharmaceutical products. The method is affordable, specific, and sensitive for routine analysis of gelatin and gelatin-containing food and pharmaceutical products to protect consumer's religious beliefs.

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