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Molecular Identification of Gelatin Origin in Pastilles and Jelly Products Collected from Tehran Markets

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ABSTRACT: Gelatin is a beneficial component in the structure of various foods such as desserts and pastilles. These products are most popular with children because of their visual appeal and bright colors. In this study, 30 samples of jelly products were collected from the Tehran markets and were tested for the molecular identification of the gelatin source. The gelatin used in the industry is mainly produced from bovine and porcine species. Identification and detection of the species used in the gelatin structure are required from the perspective of consumer confidence in food health safety as well as religious beliefs. A species-specific singleplex PCR reaction targeting 277 bp porcine D-loop regions was used to detect porcine species. It was found that none of the products contained porcine gelatin. Complementary experiments used with 130 bp bovine mitochondrial DNA (mtDNA) to detect bovine species showed that the collected samples, according to the label, contained bovine species DNA. Therefore, this qualitative PCR assay is a useful and effective method for monitoring gelatin species origin in food products and has the ability to identifying the DNA of the porcine in gelatin as a highly processed food product and Halal authentication.

Keywords: *Fraud Detection, Gelatin, Halal Authentication, Jelly Product, Qualitative PCR, Species Identification.*

Introduction

Gelatin is widely applied in many foods, pharmaceuticals, and cosmetics products. It has particular properties in terms of functions and as a protein source that does not contain fat. The gelatin in the Food Chemicals Codex (Anon, 2011) is defined as the product obtained from the acid, alkaline, or enzymatic hydrolysis of collagen, the chief protein component of the skin, bones, and, the connective tissue of animal (Gima, 2012). Gelatin comes from a variety of sources, especially from porcine and bovine species (Sahilah *et al*., 2012), it has been extracted from other sources such as chicken, fish, seaweed, insects, and yeast too, and hence authentication of gelatin is the essential issue. Since the use of porcine derivatives is restricted in Islam and some other religions, the detection and identification of the species in the product

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for food safety control organizations are essential.

According to the statistics provided on the Islamic Republic of Iran Customs Administration website, the amount of gelatin imported to Iran annually is about 3,500 tons. (Anon, 2020). Also, about thirty jelly production units are actively working in the country, following the information provided by the Food and Drug Administration of Iran. According to statistics published on the Technavio website, the amount of gelatin extracted from the porcine skin source was 42.17% versus 29.35% of bovine extracted gelatin, one of the reasons for this is the shorter extraction time of gelatin from porcine species which leads to higher quality product (Anon, 2020). Shorter production time, higher quality gelatin, and lower production costs increase the tendency to replace gelatin with the porcine source. In many Islamic countries despite the necessary controls for importing livestock and controlling meat carcasses, there is no established system for inspecting animal products and derivatives that are used as additives in the formulation of various food products, and there is a need for valid identification.

Fraud identification and control is a significant part of food safety management. In addition to the issue of fraud, one of the food industries and the control organizations' tasks is to consider and meet the interests and demands of different target groups. In food production, in addition to considering the nutritional points of the product, it is necessary to consider such things as ethical beliefs, religious laws, economic feasibility, and social trends (Rao *et al*., 2019), and also various food allergies, vegetarians lifestyle, and Zoonotic Diseases. Adulteration can have critical consequences on human health; it affects market growth by destroying consumer confidence (Doosti *et al*., 2014; Abbas *et al*., 2018).

So far, various methods have been introduced based on the identification of proteins, nucleic acids, and physicochemical properties. However, determining the gelatin origin is especially challenging due to the similarity in the amino acid sequence of collagen types in different species (Rohman *et al*., 2020). On the other hand, gelatin goes through various stages in the production process, including hydrolysis, sterilization, and drying, and this raises the possibility of destroying the structure of the material. Therfor researchers turned their attention to the development of more precise and advanced techniques.

In particular, polymerase chain reaction (PCR) in food analysis provides rapid and accurate identification of species (Girish *et al*., 2004; Kang *et al*., 2018). DNA-based methods are one of the most common methods for detecting species originality because DNA is most stable (Giovanni *et al*., 2004), if the food product is exposed to high pressure and heat processes, the proteins will be degraded or decomposed, while DNA is resistant to physicochemical factors, unlike protein biomarkers. DNA contains species-specific information and indisputably describes biological diversity, and is an ideal target for molecular detection and identification of species-specific biological products (Wang *et al*., 2018). These methods have shown a lower limit of detection (Cammà *et al*., 2012). Due to the presence of DNA in any tissues, it is possible to extract it from all kinds of tissue (Aida *et al*., 2005; Song *et al*., 2017), and only a single copy of desired DNA fragments is required for PCR amplification (Mane *et al*., 2012). In other words, these methods are accurate, stable, and allowing analysis of processed and heat-treated food products (Aida *et al*., 2005; Wang *et al*., 2018).

Various types of PCR-based methods for species authentication in gelatin have been introduced, such as PCR-RFLP (Sultana *et*

al., 2018-a), species-specific PCR (Lee *et al*., 2016), duplex PCR platforms (Nikzad *et al*., 2017), Multiplex PCR (Sultana *et al*., 2018-b) and real-time PCR (Tasara *et al*., 2005; Demiran *et al*., 2012). In order to perform the identification process of different species such as fish, domestic and wild birds, animals and especially pig, D-loop (Che Man *et al*., 2007), 16S rRNA (Dalmasso *et al*., 2004), cytochrome b (Che Man *et al*., 2012), cytochrome C oxidase I (COI) are the most commonly used markers. The use of mitochondrial DNA (mtDNA) sequences offers a series of advantages over other genetic markers (Aida *et al*., 2005; Sahileh *et al*., 2011; Fajardo *et al*., 2010). 1) There are approximately 104 copies of mtDNA available per cell in porcine species compared to only one copy of genomic DNA (Sahilah *et al*., 2011). 2) Utilization of mtDNA increases PCR amplification sensitivity because there are several copies of mtDNA per cell (Fajardo *et al*., 2010). 3) Mitochondrial genes evolve much faster than nuclear ones and, thus, contain more sequence diversity facilitating the identification of phylogenetically related species (Fajardo *et al*., 2010). 4) MtDNA is more stable because it is present in a higher number per cell (800-1000) and surrounded by a double membrane (Girish *et al*., 2004). 5). Thus it is more efficient to detect species-specific DNA using mtDNA than genomic DNA. Conserved sequences were used for species-specific primer designing. A primary aspect of successfully indicating a species by PCR is to choose suitable genetic markers to develop the assay (Fajardo *et al.*, 2010).

The present study aimed to evaluate the efficiency of the selected primer from the D-loop gene on jelly products collected from Tehran and to investigate the origin of the species and the accuracy of their labeling. For this purpose, the conventional PCR using species-specific primers was used because this method is fast, easy, low cost, and the required equipment is available in most laboratories. The result is expressed as the presence or absence of the desired target.

Materials and Methods

- Sample collection and preparation

Pure porcine species DNA was provided by the National Standards Organization of Iran, used as a reference. Thirty samples of jelly-products, including pastilles (12), marshmallows (2) jelly powder (10), and gelatin (6) produced by various companies in Iran and other countries, were prepared from shopping centers of Tehran.

- Species-specific primers

The porcine-specific primer sets used in this study were published by Mane *et al* **(**2013) based on the D-loop region of porcine mitochondrial gene sequences and 277 bp. Also, a pair of designed primers for bovine species based on ATP6 was used to complete the target. The nucleotides of the two specious-specific primers used in this study are shown in Table 1.

Table 1. The characteristics of porcine and bovine primers

Species	Primer sequences 5´-3´	Product size (bp)	Genes	Ann. temp. (^{o}C)	Reference
Porcine	F: 5' GCA AAC CAA AAC GCC AAG TAC T 3' R: 5' GGT GGT GAT ATG CAT GTT GAC TG 3'	277	D-loop	66	Mane *et al* 2013
Bovine	F:TCACAATCCAGAACTGACACC R:ACGATAAGGGTTACGAGAGGG	130	ATP6	67	Designed by Danagene lab.

- DNA extraction, evaluation of quality and purity

The steps were performed with the Danagene kit (Danagene, Iran) during cell lysis, isolation, and sedimentation steps. Extraction steps were performed according to the protocol. Also, for five of the collected samples, the extraction operation was repeated according to the protocol of the Azma gelatin kit (Elixir Azma, Iran) to compare the extraction results. Then, for quantitative analyses of the extracted DNA, a nanodrop spectrophotometer (epoch, BioTek, USA) was used for reading concentration and the adsorption at 260 and 280 nm. The extracted DNA was stored at 4 °C until use.

- PCR amplification

Conventional PCR was conducted in a total volume of 20 µL containing 2 µl Taq polymerase (yektatajhiz Inc., Iran), 4 µl 5X PCR Buffer (yektatajhiz Inc., Iran), 0.64 µL MgCl2 (yektatajhiz Inc., Iran), 0.3 µl deoxynucleoside triphosphates (dNTPs) (yektatajhiz Inc., Iran), 0.2 µl of each primer (sinaclon, Iran), 1 µl template DNA and sterile distilled water. The PCR reactions on a thermocycler (Bio Rad ،C1000, USA) were performed as follows: initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 66 °C and 67 °C for porcine and bovine respectively for 30 s and extension at 72 °C for 30 s. Then the final extension was done at 72 °C for 5 min. The amplified products of PCR were analyzed using 2% agarose gel in 1X TBE buffer (Tris-borate-ethylenediaminetetraacetic acid) and run for 30 min at 150 V. The PCR product was kept at -20 °C for further use.

- Statistical analysis

Statistical analysis was tested using SPSS version 21.0 (SPSS Inc.) and was analyzed using t-Test analysis.

Results and Discussion

Gelatin produced from porcine species has a lower price than beef species and is obtained in a shorter process thus, it is possible to use it instead of bovine gelatin as a cheaper raw material, and this indicates the need for inspection. According to the labeling requirements, the animal species, used in the product must be identified, and its labeling should be declared, on the package. The labeling content should be consistent with the sample content and verified.

Whereas gelatin is exposed to acid and alkaline hydrolysis, high-temperature extraction, sterilization, and drying during production, the proteins and nucleic acids in gelatin are highly degraded (Demiran, 2012). Most of the species-specific PCR systems tested were able to discern species of origin for tissue-derived DNA but not for gelatin DNA templates. Although this result was not unexpected and is probably linked to extensive target DNA degradation in gelatin, it indicates that the majority of species-specific PCR assays applied to other food samples might not be ideal for use with gelatin (Tasara *et al.*, 2005). The increasing probability of DNA degradation into short fragments caused some difficulties in PCR amplification as reported by earlier researchers (Mafra *et al*., 2008; Shabani *et al*., 2015).

In order to solve this problem, first, the appropriate extraction method must be used to obtain a sufficient amount of sample DNA during the extraction process, and second, the selected markers should have the ability to differentiate and authentication the species despite the small DNA fragments.

In this experiment, to identify the porcine species in highly processed gelatin products, at first, the DNA extraction step was performed using a commercial kit (Danagene kit). The results were analyzed using quantitative methods. In order to compare the extraction results, five samples

were selected from the available samples consist of two packets of pastilles, marshmallow, jelly powder, and gelatin powder then, DNA extraction, was performed with Azma kit. The concentration and purity of DNA were determined by nanodrop too. In order to compare the two methods, the values of DNA concentration as well as adsorption at 260 and 280 nm and DNA purity, were measured. The results are presented in Table 2.

Table 2. Comparison of average DNA concentration and purity of DNA obtained from two kits

Extraction method	Number	Average DNA concentration	Average DNA purity
Dana	5	16.745±1.67	1.838±0.084
Azma	5	10.44±1.56	1.592±0.072

The quality and purity with considering that the ratio of 260.280 of DNA obtained from Kit Dana, of most samples, were in the appropriate range, so the method is suitable for extraction. Differences in the amounts of extracted DNA might be due to the higher degree of denaturation and degradation of the DNA under extensive heat treatment (Ali *et al.*, 2015). The amount of extracted genomic DNA yields depend on the quantity of starting material, sample status (raw, processed, heat or chemically treated, and so on), kit, and extraction protocols. Typically, each kit is dedicated to extracting a specific substance based on protein and other components.

According to the statistical results, in comparison, both kits were successful in extracting sufficient amounts of DNA. In terms of extraction efficiency, there is no significant difference in the efficiency values of the two kits (P-value > 0.05). Azma Kit is less efficient in removing contaminants and proper DNA extraction, and the two kits are significantly different in terms of the purity of the extracted DNA (P-value < 0.05). Therefore, according to the results, the amounts of DNA obtained from both kits are sufficient and species detection with both Kits is possible.

In the next step, and to select an efficient and successful marker in identifying porcine species in highly processed jelly products, the PCR reaction was performed by 277 bp primers based on the D-loop region of mitochondrial gene sequences of porcine. These primers have previously been successful in identifying porcine species in the authentication of meat and meat products (Mane *et al* 2013). The choice of primer was for the following reasons: 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; Shabani *et al*., 2015). Shorter targets would be thermodynamically more stable than longer ones under natural decomposition and food processing treatments (Ali, 2015). The success of the D-loop gene region has already been proved by Che Man *et al*. (2012) and Kang *et al*. (2018). Kang stated that the D-loop region is capable of detecting up to 0.05% of porcine gelatin in the matrix and can effectively bind to fragmented DNA, making it suitable for detecting porcine gelatin in highly processed foods (Kang *et al*., 2018).

A Conventional PCR reaction was performed with all samples. The results showed that all of the tested jelly products were negative for porcine DNA and contained bovine DNA according to the information on the label. The gel electrophoresis results revealed expected bands of 277 bp for positive control (Figure. 1)

All the samples were tested with the introduced bovine primer. In all samples, the expected band of 130bp was observed, which indicates the presence of bovine species, and the results confirmed the absence of porcine species in the collected samples (Figure. 2)

Five samples extracted with the Azma kit were measured by PCR reaction for both 277 and 130 bp primers (Figure. 3)

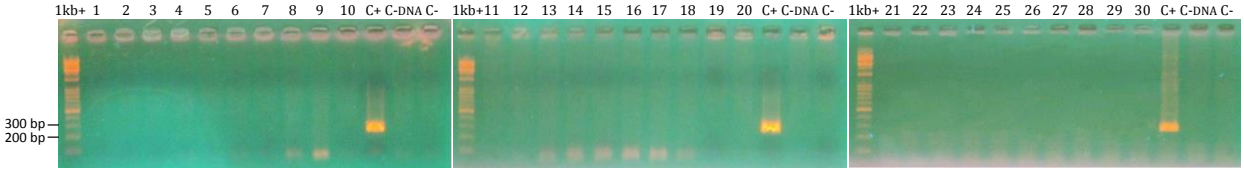

Fig. 1. PCR test result of 30 gelatin samples (277- porcine primer), 1kb+: DNA ladder, Line 1-30: Samples 1 to 30, Line C+: Positive control, Line C-DNA: DNA control, Line C-: Negative control.

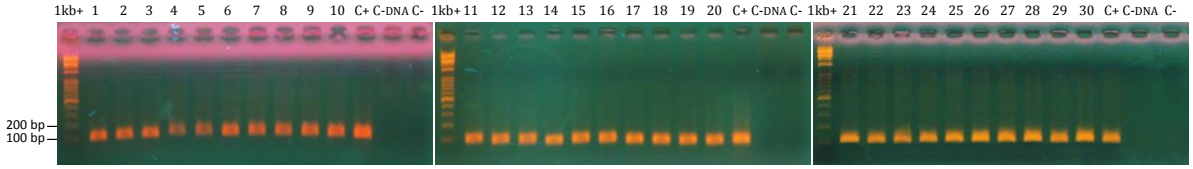

Fig. 2. PCR test result of 30 gelatin samples (130- bovine primer), 1kb+: DNA ladder, Line 1-30: Samples 1 to 30, Line C+: Positive control, Line C-DNA: DNA control, Line C-: Negative control.

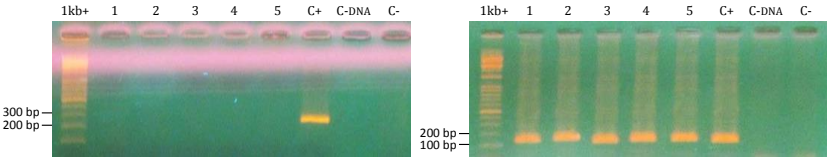

Fig. 3. PCR test result of commercial gelatin samples with Azma kit and 277 & 130 bp primers, 1kb+: DNA ladder, Lines 1 to 5: Samples 1 to 5 - Line C+: Positive control - Line C-DNA: DNA control - Line C-: Negative control.

This result is consistent with previous research by Shabani *et al*. (2015). They performed a qualitative PCR, on 16 gelatin samples, and reported that all of them were bovine origin gelatin, and absence of porcine gelatin was confirmed (Shabani *et al*., 2015). While Nikzad *et al*. (2017) performed a Duplex PCR test on 24 medicine capsules, they reported that 50% of the samples are both pure porcine gelatin and in combination with bovine and porcine gelatin (Nikzad *et al*., 2017). In 2012, Demiran *et al*. Tested 43 gelatin samples from Turkey and Germany to verify the gelatin source, including marshmallows, soft and fruity chew confectionery (gumdrops), Turkish delight, jelly, and cakes and two of fourteen samples from Germany, and one of 29 collected samples from Turkey, were contained porcine gelatin((Demiran *et al*., 2012). Soltana *et al*. (2018) performed a multiplex PCR test to differentiate between gelatin from bovine, porcine, eukaryotes, and fish in 38 Halal branded confectionery items comprising chewing gummy, marshmallows, candy, and pastilles were procured from Malaysia. Among the collected products, 33, 2, and 3 of them yielded expected bands for bovine, porcine, and eukaryotes, respectively (Soltana *et al*., 2018). Also, Soltana *et al*. (2020) studied the quantitative PCR to differentiate bovine, porcine, and fish species in 35 Halal branded food products and nutritional supplements containing gummy sweets, dietary supplements, candy and pastilles, edible gelatin powders, jellies and puddings, and yogurt containing gelatin. Out of 35 samples, only two samples contained gelatin from porcine (Soltana *et al*., 2020).

Conclusion

In this research study, in order to evaluate the origin of the species in the retail products

collected from Tehran, including gelatin powder, pastilles, marshmallows, and jelly powder, the method of conventional PCR was accompanied by 277 bp primer based on D-loop region of the porcine species gene was used. According to the PCR result for detecting porcine DNA, the samples were negative. Also, PCR analysis for detecting bovine DNA showed all collected samples contained bovine gelatin and can be used in the Iranian market. Therefor the proposed method is a reliable procedure for monitoring and certifying gelatin products and differentiate low quantity of DNA in gelatin-containing food products and can be presented to the global Halal market with the aim of authentication of the porcine species.

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