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Design and Validation of Short-Amplicon Length PCR Assay for the Detection of Porcine Gelatin in Commercial Candy and Marshmallow Products

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Abstract

Porcine gelatin has been used in many food products. To ensure the compliance of food products with halal regulations and label description, reliable analytical methods are required. In this study, Porcine-specific short amplicon-size for conventional PCR assay was developed for the detection of gelatin in commercial candy and marshmallow products. A pair of porcine-specific primers defining a 74 bp region of the mitochondrial D-loop sequence were designed. One hundred and three samples including seventy six samples from candies and twenty seven samples from marshmallow were purchased from local market in Amman, Jordan. The results revealed that seventeen samples were found to contain porcine gelatin ingredients and the rest 86 were found to be negative. Nine of the positive samples were Gelatin labeled, and eight were not identified to contain gelatin. Of these samples. Five were also labeled as Halal. So, we demonstrated that the authentication of commercial sweet ingredients using PCR assay is effective and protect consumers from being mislead due to mislabels.

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Introduction

Food authentication is one of the critical issues in food processing, which verifies that a food is in compliance with legal requirements of label. Among these requirements. Production methods, the origin or processing technologies, declaration of food origin are also important for food quality, food safety and consumer protection, as well for the compliance with national and international legislation, and guidelines. Adulteration has been a major concern of producers, consumers, and regulators since ancient times. Authenticity of food products is a major concern for safety and economic fraud reasons.

Gelatin is one of the most common food ingredients added to a wide range of food products, such as jellied desserts and meat products. Its main use is the production of candies, but is also added to a range of dairy processing. Gelatin is also widely used as a stabilizer for frozen desserts, ice cream and other as well as in cosmetic and pharmaceuticals products. It is widely used in bakery products and patisseries. Gelatin and its derivatives is also applied in other food industries of gummy desserts, toffees, chewing gum, butter, chocolate, marshmallow, soft and hard candy.[1]

Consumption of sweets in human diet is very old. Now candies are consumed by wide spectrum of population with different ages. Because they are used by children, their quality should be controlled. The ingredients of candies and sweets are very complicated and often includes many colors, flavors and preservatives. Many products like soft and hard jelly-based candy contain gelatin, which is frequently made from pig and cow. Gelatin from animal sources in food products may lead to ethical or religious issues among consumers, such as vegetarians, Halal-Kosher consumers, and consumers who should be informed about their choice. Declaration about the ingredients of food products is important for overcoming some consumer's health issues. Therefore, information about the ingredients in commercial candies is necessary for consumers to know what they are eating and help them to make ethical and safe choices.[2]

Several analytical methods have been proposed for the detection of pork gelatin or its derivatives through analyses of DNA and/or proteins. Protein-based species determination often relies on methods such as High performance liquid chromatography (HPLC) combined with fluorescence detection,[3] enzyme-linked immunosorbent assay (ELISA)[4,5] mass spectrometry,[6] Fourier transform infrared (FTIR) spectroscopy,[7] and Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis.[8]

However, gelatin as well as highly processed food products, the extreme temperature and pH treatments during production may hinder protein based analyses by progressive denaturation of the protein.[9] Moreover, such methods also proved to be unsuitable to differentiate gelatin mixture in food products.[10] On the contrary, DNA based techniques using PCR are more reliable. Nuclear or mitochondrial gene markers are quite common. However, the use of mitochondrial genes are mostly used and convenient because mitochondrial DNA extraction is much easier due to the occurrence at high copy numbers.[11] Moreover, mitochondrial DNA may also survives better in highly processed food products under environmental challenges and extreme conditions during processing[10,12]

Table 1: Summary of the candy and marshmallow samples used in this study.

Product	Gelatin labeled		Without gelatin labeled		Total
	Without halal label	Halal labeled	Without Halal labeled	halal label	
Candy	26	14	33	3	76
Marshmallow	10	16	1	0	27
Total	36	30	34	3	103

Table 2: Summary of Positive porcine ingredients detected in candy and marshmallow samples used in this study

Product	Gelatin labeled		No gelatin Label		
	no halal label	Halal labeled	no halal label	Halal labeled	Total
Candy	4	2	6	1	13
Marshmallow	1	2	1	0	4
Total	5	4	7	1	17

Other PCR based methods include species-specific conventional PCR10, SYBR green real-time PCR[11], EvaGreen real-time PCR[12] and TaqMan probe real-time PCR.[13] However, the high cost of real-time equipment and its reagents may limit their use in many laboratories around the world.

The processing operations of gelatin and gelatin containing food products, results in the degradation of DNA into short fragments.[10] In this regards, it is recommended to the use of short amplicon-length PCR assays (less than 150 bp) for the authentication target DNA in processed food products.[13,14] Therefore, the objective of this study was to develop and validate a short amplicon-size for conventional PCR assay applicable for the detection of porcine gelatin in commercial candy and marshmallow products.

Materials and Methods

Samples Collection

Pure pork meat as a positive control and one hundred and three samples consisted of seventy six samples from candy and twenty seven samples from marshmallow were purchased from local market in Amman, Jordan (Table 1). The samples were listed into two main categories. First, gelatin labeled and without gelatin labeled. Each category was subdivided into: without halal label or Halal labeled (Table 1). All samples were hygienically packed and individually opened.

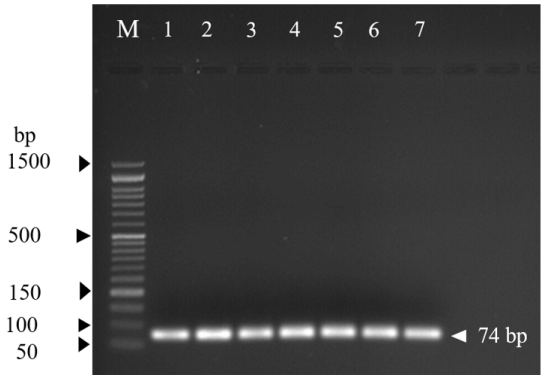

Fig.1. Agarose gel electrophoresis from gradient PCR amplification. Lane M: Marker (50 bp ladder); Lane 1: 50°C; Lane 2: 52°C; Lane 3: 53.9°C; Lane 4: 56.3°C; Lane 5: 58.3°C; Lane 6: 59.4°C; Lane 7: 60°C.

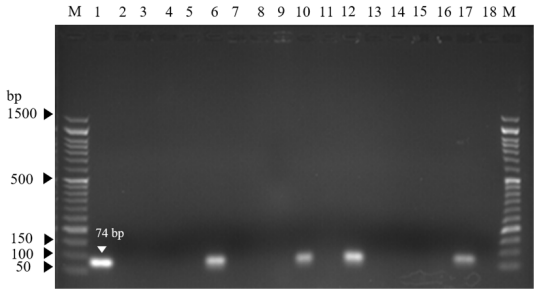

Fig. 2: Agarose gel electrophoresis of PCR products targeting porcine mitochondrial D-loop sequence. Lane M: Marker (50 bp ladder); Lane 1: Positive control; Lane 2: Negative control; Lanes 3-16: candy and marshmallow product samples collected from Jordanian market.

DNA Extraction

The total DNA was extracted from 1 gm of each candy sample following manufacturer's protocol of Wizard® SV Genomic DNA Purification System (Promega, Madison, USA). The concentration and purity of DNA were measured at 260 and 280 nm wavelengths using NanoDrop spectrophotometer (ND1000, NanoDrop thermos Fisher Scientific Inc., Ma, USA), and the extracted DNA of the samples were stored at -20ºC until further use15.

Primer design

Sus scrofa mitochondrial D-loop sequence information (GenBank accession No. JN601072.1) was downloaded from the National Center for Biotechnology Information (NCBI) GenBank. A pair of porcine-specific primers defining a 74 bp region of the mitochondrial D-loop sequence were designed using the publicly available Primer3 software (http://frodo.wi.mit.edu/primer3/). The sequence of the forward primer was
5'-CCCCCCATTAAACTTATGCTCTAC-3',
and the reverse was
5'-TTTGTACACTCTGCTTTGTTTTTGG-3'.

Conventional PCR Amplification

PCR reactions were carried out in GeneAmp PCR System 9700 (Perkin Elmer, Applied Biosystems) with a 40 µl final reaction volume containing 25 µl type-it PCR master mix (QIAGEN, Germany) mixed with 200 nM primers and genomic DNA (200–300 ng). The reaction conditions were as follows: initial denaturation step for 3 min at 95 °C, followed by 45 cycles of amplification with denaturation at 95 °C for 20 s, annealing at 60 °C for 45 s and an extension at 72 °C for 45 s, and a final extension step at 72 °C for 5 min. The optimal annealing temperature for the primer pairs was determined using a temperature gradient PCR ranging from 50°C to 60 °C. All runs included sample with target pork DNA as a positive control and also included sample without target DNA as a negative control. The PCR products were analyzed in 1.5% agarose gel by gel electrophoresis system (Cleaver Scientific, UK.; Hoefer Inc, San Francisco) containing 0.5X TBE Buffer (Tris-Base, Boric acid, EDTA buffer) at a constant voltage of 150V for 50 min and stained with ethidium bromide (10 ng/ml). The relative molecular weight of the amplified PCR products was calculated against a 50 bp ladder (GeneDirex Inc.) (Zhang, *et al.* 2009).[16]

Results and Discussion

Our newly porcine-specific primers were designed to amplify 74 bp amplicon of the D-loop sequence (accession number: FM244467.1) of mitochondrial genome from base positions 1016 to 1090. Thus, even degraded DNA molecules in the processed food products by the thermal, chemical and/or physical processes, can be detected.[17,18] To optimize the PCR conditions a gradient PCR was run utilizing annealing temperature varying from 50 °C to 60 °C for the primer combination to find the best annealing temperature (Fig. 1 & Fig. 2).

Porcine DNA was detected in seventeen samples, whereas of a total 103 samples from candies and marshmallow, 86 were found to be negative (Table 2). Our observations were similar the survey conducted by Demirhan *et al.* (2012), which was undertaken of forty-three processed food products in the Turkish market such as gum drops,[18] marshmallows, Jelly and Turkish delight. They found that two food products purchased from Germany and one product from Turkey contained porcine DNA. Moreover, in a study conducted by Sahilah *et al.* (2012) to detect the porcine DNA in gelatin containing pharmaceutical products of soft and hard gel capsules in the Malaysian market using PCR and southern-hybridization on the biochip analysis. They found that 42 of the 113 soft and hard capsule sample tested contained porcine DNA.[1]

Ten samples from candy and two samples from marshmallow contained porcine DNA and did not show Halal on their label. On the other hand, three candy and two marshmallow samples contained porcine DNA from products were indicated on the product label as Halal. In some markets, porcine DNA have been detected in a number of food products in spite of being halal labeled on packaging. For example a study conducted by Farouk *et al.* (2006) showed that 2 food products (chocolates) which were labeled as halal were positive for porcine ingredients in the Malaysian food market.[19] Another study was performed on thirty three food samples labeled as halal in the Saudi market, where two

imported samples of beef sausage and beef steak were positive for porcine contaminants.[20]

Eight samples from candy and Marshmallow not labeled as containing gelatin were found to be positive tor porcine DNA in the current study (Table 2.). Porcine DNA traces have also been detected in European candy products despite being labeled as vegetarian.[2,21,22] Therefore we recommend the authentication of commercial sweet ingredients using PCR assay, to protect the consumers from adulteration and mislabeling which can affects their eating habits.

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Conflict of interest

The author declare no potential conflict of interests.

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