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Identification and verification of porcine DNA in commercial gelatin and gelatin containing processed foods

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ABSTRACT

Gelatin, derived from bovine and porcine sources, has been used in many foods and pharmaceutical products. To ensure the compliance of food products with halal regulations, the reliable analytical methods are very much required. In this study, polymerase chain reaction (PCR) assay using species-specific primers was performed to evaluate the halal authenticity of commercial pure gelatin and gelatin-containing processed food products. Based on the specificity and cross-reactivity results of the seven species-specific primers by conventional PCR, the porcine species primer No. 2 was selected and it was able to detect species DNA in 12 out of 36 processed foods. The cloning, sequencing, and blasting at NCBI confirmed the presence of pork DNA in 5 out of 12 porcine DNA positive food samples. The maximum identity (homology) with pork sequence available in NCBI Gene Bank for the five samples ranged from 87% to 97% and the Query Cover ranged from 94% to 100%. The real-time PCR assay detected more positive samples (27 positive amplifications) compared to 12 positive samples with conventional PCR using porcine specific primer No. 2. PCR using species specific primers is a very useful and effective technique for halal authenticity of gelatin and gelatin-containing food products.

1. Introduction

Muslim Halal (purification) law requires food and non-food products free from pork and its derivatives. The Halal food can easily make up to 20% of word trade in food products as Muslims are expected to represent 30% of the world's population by the year 2025 (Karim & Bhat, 2008).

Gelatin is produced by the selective hydrolysis of collagen, the main protein in skin, hides, white connective tissues, and bones of the animal body (Tasara, Schumacher, & Stephan, 2005). It is the most widely used pig derivative ingredient in production of processed foods. Gelatin is classified as a foodstuff, with E number (E441) and can be used at concentration ranging from 0.25% in frozen pies to 0.5% in ice cream. Other products like gummy candies, canned ham, various luncheon meals, corned beef, chicken rolls, jellied beef, margarine, sour cream, cottage cheese, lozenges, wafers, marshmallows, gelatin dessert, and some low-fat yogurt, may contain up to 1–5% gelatin (Keenan, 1997).

The beginning use of the PCR for the detection of DNA source was in Europe as a response to the Bovine Spongiform Encephalopathy crisis, and when non-Muslim consumers wanted to eat pork gelatin free of bovine gelatin. The identification of different animal species has been done by detecting DNA sequences but it is limited due to the single-copy nature of many of the sequences (Walker et al., 2004). Tasara et al. (2005) indicated that the technique can work in highly processed food products because of the greater stability of DNA compared with proteins. Montiel-Sosa et al. (2000) designed highly species-specific primers for pork D-loop mtDNA using restrictive PCR amplification conditions, and developed a fast and reliable method for detecting a PCR-amplified 531 bp fragment from pork. The technique was suitable for detecting both pig meat and fat in meat mixtures, including those dry-cured and heated samples.

The food industry and mass market globalization might allow for cheaper or prohibited ingredients and consequently mislabeling or fraudulent substitution or deceptive labeling (Amaral, Santos, Oliveira, & Mafra, 2017). The detection of porcine DNA in food products has been a great challenge to researchers, food processors,

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food regulators and food industry due to lack of reliable methods of detection particularly in processed foods.

According to labeling requirements, the source of gelatin must be clearly and truthfully identified and declared on the food package and any ambiguity in the labeling practices is unacceptable. About 80% of edible gelatin in Europe is derived from pig skin, and about 15% comes from the hide splits of bovine origin. The remaining 5% is of bone origin, extracted from poultry, bovine, porcine, or fish species (Boran & Regenstein, 2010; Demirhan, Ulca, & Senyuva, 2012; Tasara et al., 2005). Some commercially produced fish gelatins are available, but are not commonly utilized because of their inferior rheological properties compared to mammalian gelatin, which affect product quality (Cho, Gu, & Kim, 2005).

There are a limited number of reports available on animal species-specific identification of gelatin, gelatin-containing foods, and pharmaceutical products using species-specific primers by PCR techniques (Cai, Gu, Scanlan, Ramatlapeng, & Lively, 2012; Demirhan et al., 2012; Mutalib et al., 2015; Shabani et al., 2015; Tasara et al., 2005). Therefore, the present study was aimed to perform conventional PCR using a set of species-specific primers for Halal authentication of commercial pure gelatin and gelatin-containing processed food products. Furthermore, the sensitivity of Real-time PCR and conventional PCR was compared in verifying the porcine DNA present in processed foods having gelatin label.

2. Materials and methods

2.1. Materials

2.1.1. Food samples

Thirty-six commercial processed food samples (two pure edible gelatin, 12 jelly, nine marshmallows, six candies, two chewing gums, one gummy pizza, one milko mix toffee, one salsa, one mallow cake and one medicine tablet) from 15 different countries of origin, were collected from hypermarkets in Riyadh, Saudi Arabia. Among the samples, 33 products showed gelatin on their label ingredients list, whereas two processed food samples and the medicine tablet sample did not have gelatin on the label.

Canned pork (porcine) was brought from a Malaysian market in Kuala Lumpur (country of origin was China). Chicken, beef (bovine), and fish samples were purchased from supermarket in Riyadh, Saudi Arabia. Meat tissue samples were used for isolation of species-specific DNA as positive control.

2.1.2. Primers and kits

Seven different species-specific PCR primer sets from published literature are illustrated in Table 1. Primer specificity for tissue or processed food-derived DNA templates was evaluated by conventional PCR. The primers targeted 134, 290, and 152 bp fragment in the mt cyt *b* gene of pig.

Real Time PCR kit (SYBR® Green, PCR Core Reagents, Applied Biosystems™, UK), Cloning kit (Promega, pGEM-T Easy Vector, System I) and sequencing kit (Applied Biosystems BigDye® Terminator v3.1) were used for the analysis work.

2.2. Methods

2.2.1. DNA extraction and purification

DNA extraction and purification from meat tissue and processed food samples was carried out according to the method of Milligan (1998).

2.2.2. Thermocycler (conventional PCR)

The Mastercycler, Nexus Gradient (Eppendorf GA, 22331, Hamburg, Germany) was used for this study. The reaction mixture consisted of 10 μl of Go Taq Green Master Mix (2×, REF M712c, Promega, USA), 1 μl $MgCl_2$ (25 mM), 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 4 μl DNA, and 3 μl DNase free water. Gradient PCR was carried out and the amplification conditions for all primers were as follows; an initial denaturation for 5 min at 94 °C followed by 10 cycles for 45 s at 94 °C, 45 s at annealing temperature specific to each primer as given in Table 1, and for 1 min at 72 °C, then 25 cycles at 94 °C for 45 s, 42 °C for 45 s and 72 °C for 1 min, and final extension at 72 °C for 25 min.

2.2.3. Electrophoresis and gel purification

The conventional PCR products were analyzed by electrophoresis in 2% agarose gel in trice boric acid-EDTA buffer, stained by acridine orange, and visualized after 25 min by 70 V electrophoresis machine (Cleaver Scientific Ltd. UK). The gel document was captured by Gel Doc™ EZ Imager, (BIO-RAD, USA). The fragments of positive samples were cut by scalpel and the amplicons were purified from the gel by using EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic, Canada).

2.2.4. Cloning and sequencing

Cloning and sequencing were performed for confirmation only for processed foods which showed positive amplification with the pork primer No.2 using conventional PCR. The cloning was carried out using Promega Kit (pGEM®- Easy Vector System I Madison, WI., USA), according to the protocol of the manufacturer. Dye-terminator (Applied Biosystems Big Dye® Terminator v3.1) sequencing reactions were performed in a thermal cycler (model: FTC5, Bibby Scientific Ltd. UK), according to the protocols supplied

Table 1
Species-specific primers sequences and amplification conditions.

Primer No.	Sequence (5′–3′)	Specificity	Annealing temperature (°C)	Amplicon size (bp)	Target gene	Reference
1	GGTTTACGACCTCGATGTTG (F) CGGGTCTGAACTCAGATCAC (R)	Universal	55	104	16S rRNA	(Tasara et al., 2005)
2	GACTAGGAACCATGAGGTTGCG (F) AGCCTACACCACAGCCACAG (R)	Porcine	58	134	PRE-1 SINE element	(Tasara et al., 2005)
3	CTACATAAGAATATCCACCACA (F) ACATTGTGGGATCTTCTAGGT (R)	Porcine	55	290	12S rRNA-tRNA Val	(Tasara et al., 2005)
4	ATGATCTTATCAATATTCTTGACCC (F) CCTTCAAGGGGTGTTTTGTTTTAA (R)	Bovine	55	126	ATPase 8 subunit	(Tasara et al., 2005)
5	GGGACACCCTCCCCCTTAATGACA (F) GGAGGGCTGGAAGAAGGAGTG (R)	Chicken	58	266	tRNA-Lys	(Lahiff et al., 2001)
6	CCTTGCYHAGCCACACCCC (F) CGTATAACCGCGGTGGCT (R)	Fish	60	121–124	mitochondrial 12S ribosomal RNA (12S rRNA)	(Benedetto et al., 2011)
7	TGCAGTCTGTCTCCTCCAAA(F) CGATAATTGGATCACATTTCTG (R)	Pork	55	152	12S rRNA	(Farouk et al., 2006)

F: forward; R: reverse.