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Detection of porcine DNA in gelatine and gelatine-containing processed food products—Halal/Kosher authentication

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ABSTRACT

A commercially available real-time PCR, based on a multi-copy target cytochrome *b* (cyt *b*) using porcine specific primers, has been validated for the Halal/Kosher authentication of gelatine. Extraction and purification of DNA from gelatine were successfully achieved using the SureFood® PREP Animal system, and real-time PCR was carried out using SureFood® Animal ID Pork Sens kit. The minimum level of adulteration that could be detected was 1.0% w/w for marshmallows and gum drops. A small survey was undertaken of processed food products such as gum drops, marshmallows and Turkish delight, believed to contain gelatine. Of fourteen food products from Germany, two samples were found to contain porcine gelatine, whereas of twenty-nine samples from Turkey twenty-eight were negative. However, one product from Turkey contained porcine DNA and thus was not Halal, and neither was the use of porcine gelatine indicated on the product label.

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1. Introduction

Gelatine is a highly processed protein, which is widely used as a gelling and thickening agent (E441) in a variety of foodstuffs such confectionary products, water-based desserts and in the pharmaceutical industry e.g. in gel capsules for medicines. Gelatine is obtained by hydrolysis of collagen, which is extracted from materials such as bones, hides and skins from animal slaughterhouses (Karim and Bhat, 2008). Gelatine production involves controlled acidic or basic hydrolysis of connective tissue raw material, high temperature extraction with water, sterilisation, and drying. These processes are not standardised and have effects on the properties of the final gelatine product. In the final gelatine product, both proteins and nucleic acids are highly degraded (Boran and Regenstein, 2010). Additionally, the amount of DNA in gelatine is very low and differs from material-to-material.

In Europe, about 80% of edible gelatine is produced from pigskin, but vegetarian, Halal and Kosher gelatine, prepared from seaweed, fish bones or non-porcine sources, is also available (Boran and Regenstein, 2010). Although gelatine must be labelled appropriately, once it has been manufactured, purified and in commercial trade, it is difficult to ensure its provenance or whether it has been inadvertently mixed at any point in the food chain. It is therefore important to have methods available whereby pure gelatine can be checked to ensure its authenticity and that it is free from cross-contamination with porcine gelatine. Equally the ability to test processed food products for the presence of porcine gelatine is an essential requirement for food control in Muslim or Jewish communities (Riaz and Chaudry, 2004).

Most published methods have focussed on meat species identification rather than identification of gelatine. Polymerase chain reaction (PCR)-based methods have been the most successful in terms of both specificity and sensitivity of species detection. A review of PCR-based methods applied to the authentication of meat products cites some twenty-nine publications (Mafra, Ferreira, and Oliveira, 2008). Extraction of good quality DNA is an important pre-requisite for PCR-based analysis and this can be a potential problem if there has been extensive heat processing. For example, only poor quality genomic DNA was extractable from bread and biscuits, although it is not clear if this was because of high temperature degradation during cooking or because lard, containing only small amounts of DNA, was the target source of DNA (Aida, Che Man, Raha, and Son, 2007). DNA has been isolated from meat and cheese using a standard CTAB protocol and from milk using a Promega Wizard Magnetic kit and purified by Qiagen silicon spin columns (Zhang, Fowler, Scott, Lawson, and Slater, 2007). With gelatine, despite both extensive heat and chemical treatment, it has been demonstrated that it is possible with nucleic acid binding columns or standard ethanol precipitation to obtain template DNA. Analysis of the extracted DNA on agarose gels was used to demonstrate that it had remained essentially intact (Tasara, Schumacher, and Stephan, 2005).

A number of PCR approaches have been used to detect porcine DNA in meat and meat products (e.g. Binke, Spiegel, and Schwägele, 2007). Using restriction fragment length polymorphism (RFLP)

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analysis of a conserved region of the mt cytb DNA extracted from sausages, clear PCR products were produced on amplification (Aida et al., 2007). For the same food products, using species-specific PCR, detection of a conserved region in the mt 12S ribosomal RNA (rRNA) gene was employed as an alternative method. The extracted DNA was amplified by PCR targeting specific regions of the 12S rRNA gene of 387 base pairs (bp) from pork species. The species-specific PCR was used for successful identification of pork DNA, but the performance of the method in terms of sensitivity was not reported (Che Man, Aida, Raha, and Son, 2007).

A similar method utilising PCR–RFLP was reported for beef, pork, buffalo, quail, chicken, goat, and rabbit species identification and Halal authentication. PCR products of 359-bp were successfully obtained from the cytb gene of these meats, and five different specific enzymes were identified as potential restriction endonucleases for differentiation purposes (Murugaiah et al., 2009). Specific PCR amplification of a repetitive DNA sequence has been used for the identification of pork in processed and unprocessed food. A level of addition of 1% pork was detectable with 20 PCR amplification cycles and 0.005% pork with 30 PCR amplification cycles (Calvo, Zaragoza, and Osta, 2001). A species-specific duplex PCR assay has been used for the simultaneous detection of pork and poultry meat species, again using the mt cytb as target gene for pork. By amplification of DNA from meat mixtures of two species, linear calibration was obtained using fluorescence intensities of PCR products for pork (149-bp) in the range of 1–75%, with a sensitivity of 0.1% addition. In-house validation, using samples with known amounts of pork, gave a coefficient of variation from 4.1 to 7.6% (Soares, Amaral, Isabel Mafra, Oliveira, and Beatriz, 2010). Real-time PCR has also been used for the identification of beef, pork, horse, mutton, chicken and turkey in processed meat down to a level of 0.01–0.05% (Jonker, Tilburg, Gele, and De Boer, 2008).

TaqMan real-time PCR using a bovine-specific primer pair for the mt cyt *b* gene and a FAM-labelled mammalian-specific cyt *b* probe could quantitatively detect as little as 35 pg bovine DNA and showed no cross-reaction with ovine, caprine or porcine DNA. The system was used to measure bovine DNA in fresh and processed meat, milk and cheese (Zhang et al., 2007). Specific primers and TaqMan probes have been designed for the mt ND2, ND5 and ATP 6–8 genes for donkey, pork and horse, respectively. Only one cross-reaction was observed between the horse species specific primer-probe set and 100 ng pork DNA at the cycle threshold (Ct) value of 33.01 (corresponding to 0.01 ng horse DNA). The assay enabled the detection of 0.0001 ng of template DNA from pure meat for each species investigated (Kesmen, Gulluce, Sahin, and Yetim, 2009).

Several species-specific PCR methods have been published to determine the origin of raw materials used in gelatine manufacture. A bovine species-specific PCR primer set targeting the ATPase 8 subunit gene in bovine mt DNA was demonstrated to be suitable for detection of bovine material in gelatine. This PCR primer set was optimised using conventional and real-time PCR approaches. The inclusion of bovine gelatine in pork or fish gelatine could be detected at levels of 0.1% by conventional PCR and 0.001% by light cycler PCR after DNase I decontamination (Tasara et al., 2005). The viability of testing pure gelatine by PCR was demonstrated, although the method was not taken any further in terms of analysis of commercial gelatine-containing food products.

In this paper we have deliberately adopted the approach of using commercial test kits both for DNA extraction and for the real-time PCR analysis. Although, there have been many very successful methods published for detection of porcine DNA, there is a real need for food control laboratories to apply these methods routinely using commercially available kits. We have focussed on gelatine because this product seems to have been overlooked in terms of testing methodology, and yet has a high potential for inadvertent adulteration with porcine material or mislabelling.

2. Materials and methods

2.1. Sample preparation

Twelve gelatine samples of known origin (bovine, porcine or seaweed) were obtained in powder or sheet form, and employed as reference standards. Pure gelatine mixtures were prepared by extracting and purifying the DNA from 500 mg of porcine gelatine and diluting the resulting DNA solution with bovine DNA solution to obtain 10%, 1% and 0.1% mixtures. Mixtures of food products containing porcine gelatine were individually prepared by grinding gum drops or marshmallow together with porcine gelatine, and mixing to a fine powder. The composition of these mixtures is shown in Table 1.

Forty-three samples of the soft and fruity chew confectionery (gum drops), Turkish delight, jelly and marshmallows/cakes containing gelatine were obtained from markets in Turkey and Germany. Samples were stored at −20 °C. Approximately, 300 g of each sample was blended in the frozen state using a Waring blender (Torrington, USA) to produce a powder, which was thoroughly mixed. Subsamples (400 mg) were taken for DNA extraction.

Spiking of the above retail food products with 5% porcine gelatine was carried out by weighing a 380 mg amount of the composite product into a 1.5 ml reaction tube together with 20 mg of porcine gelatine. The whole mixture (400 mg) was then taken for DNA extraction.

2.2. Extraction of DNA

DNA was extracted from pure gelatine or from food products containing gelatine (400 mg) using the Sure Food® Prep Animal X kit (CONGEN, R-Biopharm, Germany). Lysis buffer (1000 µl) and Proteinase K (40 µl) were added to 400 mg of sample and mixed by vortexing (Fisherbrand ZX Wizard). The mixture was incubated at 65 °C for 1 hour in a thermomixer (Eppendorf, comfort) under continuous shaking. At the end of the incubation, the solution was centrifuged at 24,150*g* for 2 min (Eppendorf 5430). After centrifuging, a spin filter was placed in a receiver tube. The solution was transferred into spin filter and centrifuged at 24,150*g* for 2 min. The spin filter was discarded. Binding buffer (200 µl) was added to the filtrate, which was vortexed thoroughly. The filtrate was transferred to a new spin filter placed in a new receiver tube and centrifuged again at 24,150*g* for 2 min. After the filtrate was discarded, 550 µl of pre-wash buffer was added into the spin filter and centrifuged at 24,150*g* for 1 min. This step was repeated twice. After discarding the filter, it was centrifuged for 2 min at 24,150*g* to remove wash buffer completely. A new spin filter was placed in a new 1.5 ml receiver tube; 50 µl of preheated elution buffer was pipetted directly onto the spin filter and incubated at room temperature for 3 min. Finally, it was centrifuged for 2 min at 16,770*g* and the purified DNA solution (50 µl) was stored at 4 °C.

2.3. PCR amplification

A pork reaction mixture containing specific primers and Taq-Polymerase are supplied as part of the commercial test kit. The reaction mix, Taq-Polymerase (SureFood® Animal ID Pork SENS Plus V kit) and extracted DNA were mixed in the ratio 9.95: 0.05: 2.5 for

Table 1
Composition of mixtures of gum drops/marshmallows mixed with various levels of porcine gelatin.

Level of addition (%)	Wt of food (mg)	Wt of bovine gelatine (mg)
1.0	495.0	5.0
3.0	388.0	12.0
5.0	380.0	20.0
10.0	450.0	50.0

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