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Evaluation of DNA extraction protocols and real-time PCR-based methods for efficient investigation of pig traces in foods

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Abstract

Industrially processed foods are composed of a complex mixture of molecules combined under specific chemical and physical conditions. Besides their native interactions, most of the ingredients included in processed foods are highly transformed through extreme heat variations, grinding, freezing, pH, and pressure fluctuations in order to reach the desired final product. Due to their complex structure and high level of degradation, processed foods are difficult to analyse. Undeclared components are often detected in processed foods, and accurate diagnostic testing is required to protect those with health, cultural, and religious restrictions. Molecular biology techniques involving PCR are most frequently used for determining the authenticity of foods containing derivatives of living organisms. In the present work, we investigated four different DNA extraction protocols of three commercial kits, two different quantitative PCR (qPCR) techniques, and six different primer pairs. We analysed 96 extracts (12 samples from each of the eight products) by SYBR Green-based qPCR using the two most specific and sensitive primer pairs, and compared these results to those obtained with standard commercial kits that use dual dye-labelled probes. Adopting high-efficiency DNA extraction protocols, our findings highlighted the importance of targeting several small regions of the mitochondrial genome to effectively detect small traces of porcine products, and reduce the risk of false-negative results. Adopting these will ensure that consumers can make accurate and informed choices.

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Introduction

Food falsification has evolved from being an instance of fraudulence to a highly sophisticated business. Therefore, transparency in accurately identifying all the ingredients on the label of processed food products has become increasingly important and challenging, a particularly pressing concern among consumers for religious, health, and regulation-related reasons (Gargouri and Hadj Kacem, 2018). Communities like Islam and Judaism forbid the use of pork-based ingredients, while Hinduism prohibits the consumption of beef. Consequently, authorities have been appointed to check the quality of processed food and non-food products such as fitness, pharmaceutical, cosmetic, and leather goods (Bonne and Verbeke, 2008).

A common ingredient used widely in foodstuffs is gelatine. It is a soluble aqueous protein substance obtained by the thermal denaturation of collagen, which is mainly extracted from animal by-products like skin, connective tissues, and bones (Mariod and Adam, 2013). Gelatine has an extensive range of applications in various industries, including as food additives and gelling agents, and coded as E441. Gelatine production involves multiple extreme procedures such as the acidic or basic hydrolysis of connective tissue raw materials. Notably, these processes are not standardised, and have diverse effects on the properties of the final gelatine products (Bonne and Verbeke, 2008; Demirhan *et al.*, 2012; Sahilah *et al.*, 2012).

Various published protocols focus on finding the most sensitive method for animal identification in

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meat products rather than highly processed foods (*e.g.*, gelatines, candies, biscuits, and chips). Recent studies use molecular techniques that rely on protein and DNA analyses to investigate whether the information on food labels is accurate, and no hidden, undesirable ingredients have contaminated the final product. Consequently, protein-based analytical techniques using "immuno" assays such as enzyme-linked immunosorbent assays (ELISAs), high-performance liquid chromatography (HPLC), and matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF-MS) are suitable for the authentication of raw meats and some gelatine samples. However, these methods are unsuitable for food traceability studies due to their low sensitivity and detection limit issues arising from extreme thermal processes that can denature and alter the protein epitopes important for identification and authentication (Flaudrops *et al.*, 2015; Tukiran *et al.*, 2016; Li *et al.*, 2019; Yap and Gam, 2019).

To date, polymerase chain reaction (PCR) has proven to be the optimal technique for the authentication of animal species and their derivatives in several highly processed food products with high specificity and sensitivity (Kleinnijenhuis *et al.*, 2018). However, in the final food products, both the proteins and nucleic acids are highly degraded. Additionally, the amount of DNA in gelatine or most food ingredients is very low, depending on the specific food category (Malik *et al.*, 2016). To overcome this issue, mitochondrial DNA (mtDNA) is the best DNA marker target for most fragmented DNA. Not only it is present in multiple copies in most cells, but its circular shape makes it more resistant to extreme food processing procedures (Cammà *et al.*, 2012). Shorter DNA products (size range between 100 and 200 bp) of several mtDNA genes could increase the chance of targeting the traceability of food adulteration (Dooley *et al.*, 2004; Rahman and Hassan, 2018). Nevertheless, DNA extraction from a variety of food samples has been challenging for most researchers, even when using pure gelatine samples (Mohamad *et al.* 2016). Consequently, finding the best method for isolating DNA with high quantity and quality is of utmost importance for successful downstream analysis and accurate, reliable results (Hsieh *et al.*, 2016; Sultana *et al.*, 2018). Extracting DNA from fresh and processed meats (*e.g.*, cooked, heated, ground, uncooked, and dried) using commercial kits or standard protocols [*e.g.*, cetyltrimethylammonium bromide (CTAB), phenol-chloroform] is well-optimised as compared to from candies, jellies, and marshmallows, regardless of the origin of the gelatine (Piskata *et al.* 2019).

Quantitative PCR (qPCR) has become the cornerstone of molecular biology tools used to detect and quantify fragmented and low-quantity DNA in highly processed foods (Martín *et al.*, 2009; Sultana *et al.*, 2018). The chemistry of fluorescent dyes underlies two distinct qPCR methodologies (SYBR Green-based and probe-based) with different specificities (Cai *et al.*, 2012). Specificity is the primary concern with SYBR Green (or any dsDNA-binding dye), while higher specificity is shown by the labelled probe method. Most of the commercial kits designed for porcine gelatine detection are probe-based. However, both methods carry a similar risk of false-negative results with highly degraded DNA (Figure 1), which is unacceptable, given that the consumption of pig derivatives in any quantity or form is prohibited in the Muslim community.

Therefore, the present work aimed to evaluate and eliminate the occurrence of false-negative results by comparing different DNA extraction protocols, assessing the effect of multiplying DNA targets, and combining both qPCR methods, to overcome the limitations caused by DNA fragmentation.

Materials and methods

Samples collection and preparation

A total of eight products were analysed. Pure porcine gelatine powder was purchased from Sigma-Aldrich (St. Louis, MO, USA), and four different food samples (flavoured gelatine powder, soft candies, chips, and biscuits) labelled as containing traces of pork were purchased from a UAE market that sells non-Muslim foods (Waitrose, Dubai). Bovine gelatine powder was purchased from a supermarket in the UAE, and two soft candies were purchased from a supermarket in Norway (Table 1). Sample matrices were prepared depending on the type of sample. The gelatine powder samples were used directly; soft candies were minced using a disposable sterile scalpel, meanwhile the chips and biscuits were ground using an analytical miller (IKA® A11 basic, Germany).

DNA extraction

All DNA extractions were performed according to the manufacturer's protocols with minor modifications to maximise the recovery of short DNA

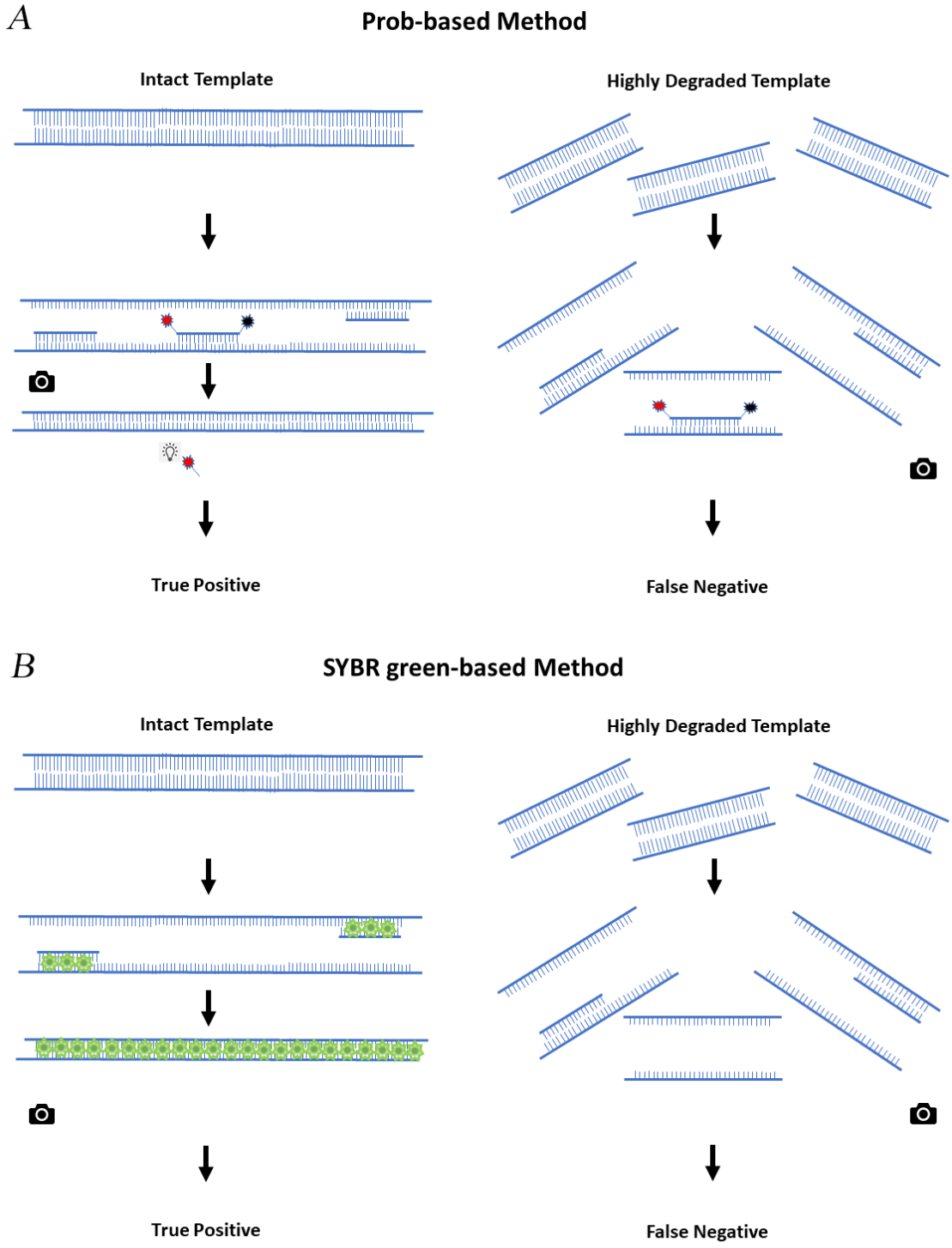

Figure 1. Illustration of the risk of false-negative results using **(A)** probe-based and **(B)** SYBR Green-based methods on highly degraded DNA template.

Table 1. Food products assessed in the present work.

No.	Sample name	Sample description	Animal source ingredient	Origin	Market	Animal source claimed by the manufacturer
1	Reference gelatine powder	Porcine skin-derived reference powder	Gelatine	USA	Sigma-Aldrich, St. Louis, MO, USA	Porcine
2	Orange-flavoured gelatine powder	Orange sugar-free gelatine dessert mix	Gelatine	USA	Waitrose Supermarket, UAE	Porcine
3	Soft candy	Jelly and marshmallow figures	Gelatine	Norway	Norway Supermarket	Not mentioned
4	Sugared soft candy	Sour jelly figures	Gelatine	Norway	Norway Supermarket	Not mentioned
5	Candy	Cherry ropes filled with punch	Gelatine	USA	Waitrose Supermarket, UAE	Porcine
6	Bovine gelatine powder	Edible bovine gelatine powder (clear, unflavoured)	Gelatine	Australia	UAE market	Bovine
7	Chips	Seasoned pork shank rinds	Pork rinds	UK	Waitrose Supermarket, UAE	Porcine
8	Biscuits	Tarts with strawberry-gelatine filling	Gelatine	USA	Waitrose Supermarket, UAE	Porcine

fragments. All extraction processes were completed under DNA-contamination-free conditions (70% ethanol, 10% bleach, and environmental DNases I) to avoid contamination from reagents, laboratory environment contamination, and cross-contamination among samples. DNA from chicken, cow, turkey, and pig peripheral blood, which were used as references, were extracted using a standard phenol-chloroform method (Kawasaki, 1990).

Protocol 1: DNeasy® Mericon® Food Kit using 2 g of samples recommended for highly processed food

DNA was isolated in a large-scale (2 g) of small-fragment protocol designed for highly processed food material. First, 2 g of sample was weighed and transferred to a 50-mL centrifuge tube. Then, 5 mL of lysis buffer was added, and the samples were homogenised for 30 s using the TissueRuptor II (Qiagen). Later, 25 µL of proteinase K solution was added to the tubes and vortexed briefly. Afterward, the samples were incubated overnight at 60°C in a dry bath incubator (VorTemp™ 1550 Labnet, USA) with constant shaking to ensure complete dispersal and saturation of the sample material. Subsequently, the solution was cooled to room temperature and centrifuged (LSE™ Corning, USA) for 5 min at 2,500 *g*. Next, 1 mL of the clear supernatant was transferred to a new 2-mL microcentrifuge tube containing 500 µL of chloroform (Merck, Germany); samples were then vortexed for 30 s, and centrifuged at 14,000 *g* for 15 min. Thereafter, 1 mL of PB buffer was pipetted into a fresh 2-mL microcentrifuge tube, and the maximum volume of the upper aqueous phase was transferred and mixed thoroughly by vortexing. Next, 650 µL of the mixture was pipetted into a spin column placed in a 2-mL collection tube, and incubated for 5 min at room temperature, then centrifuged at 17,900 *g* for 2 min; the flow-through was subsequently discarded, and this step was repeated for the remaining mixture. Subsequently, 500 µL of wash buffer was added to the spin column, and centrifuged at 17,900 *g* for 1 min; then, the flow-through was

discarded. The collection tube was centrifuged again at 17,900 *g* for 2 min to dry the membrane. Finally, the spin column was transferred to a new 1.5-mL microcentrifuge tube, and 50 µL of elution buffer was added onto the membrane. After incubating for 5 min at room temperature, the sample was centrifuged at 17,900 *g* for 1 min to elute the extracted DNA.

Protocol 2: DNeasy® Mericon® Food Kit using the standard protocol

DNA was isolated from 0.2 g of sample, and transferred to a 2-mL microcentrifuge tube. Then, 1 mL of lysis buffer was added to the sample, and homogenised for 30 s using the TissueLyser (Qiagen) for 20 s at 15 Hz. Next, 25 µL of proteinase K solution was added to the tubes and vortexed briefly to ensure complete dispersal and saturating of the sample material. Later, samples were incubated overnight at 60°C in a dry bath incubator (Thermo Scientific, USA) to maximise both the lysis and final DNA yield. Next, the solution was cooled to room temperature, and centrifuged (Sigma1-15, Germany) for 5 min at 2,500 *g*. Then, the entire clear supernatant was transferred to a new 2-mL microcentrifuge tube containing 500 µL of chloroform (Merck, Germany); samples were then vortexed for 30 s, and centrifuged at 14,000 *g* for 15 min. After that, 1 mL of PB buffer was pipetted into a new 2-mL microcentrifuge tube, and the maximum volume of the upper aqueous phase was transferred and mixed thoroughly by vortexing. Next, 650 µL of the mixture was pipetted into a spin column placed in a 2-mL collection tube, and incubated for 5 min at room temperature, then centrifuged at 17,900 *g* for 2 min; then the flow-through was subsequently discarded. This step was repeated for the remaining mixture. Subsequently, 500 µL of the wash buffer was added to the spin column, and centrifuged at 17,900 *g* for 1 min; then the flow-through was discarded. The collection tube was centrifuged again at 17,900 *g* for 2 min to dry the membrane. Finally, the spin column was transferred to a new 1.5-mL microcentrifuge tube, and 50 µL of elution buffer was added onto the membrane. After incubating for 5 min at room temperature, the tube was centrifuged at 17,900 *g* for 1 min.

Protocol 3: foodproof® Sample Preparation Kit III

DNA was isolated using foodproof® Sample Preparation Kit III (DNA isolation for identification analysis of animal species protocol; BIOTECON Diagnostics). Firstly, 0.2 g of sample was weighed out and transferred to a 2-mL microcentrifuge tube. Then, the extraction process began by adding 1 mL of extraction buffer to the samples, and homogenised for 30 s using TissueLyser (Qiagen) for 20 s at 15 Hz. Afterward, 80 µL of proteinase K in 2-mL microcentrifuge tubes was added. Later, the samples were vortex for 30 s, and incubated overnight at 72°C in a dry bath incubator (Thermo Scientific, USA), during which the samples were mixed two to three times by inverting the tubes. Afterward, the samples were centrifuged (Sigma1-15, Germany) at 12,000 *g* for 10 min. Subsequently, 400 µL of binding buffer and 200 µL of isopropanol were added to a new 2-mL microcentrifuge tube, and the maximum volume of the previous supernatant was transferred to the 2-mL microcentrifuge tube with binding buffer and isopropanol, then the samples were mixed gently by pipetting up and down. Afterward, 650 µL of the mixture was pipetted into the filter tube assembled on a collection tube, and centrifuged for 1 min at 5,000 *g*. Then, the flow-through and collection cube were discarded, and the filter was placed into a new collection tube. This process was repeated with the remaining mixture. Thereafter, 450 µL of wash buffer was added to the upper reservoir, and centrifuged for 1 min at 5,000 *g*. The flow-through was discarded, and 450 µL of wash buffer was added for a second time to the upper reservoir, and centrifuged again for 1 min at 5,000 *g*. Then, the flow-through was discarded, and the collection tube was centrifuged again to remove any residual wash buffer for 10 s at max speed (13,000 *g*). Next, the filter tube was inserted into a new 1.5-mL reaction tube, while the elution buffer was pre-warmed at 70°C in a dry bath incubator (Thermo Scientific, USA). Finally, 50 µL of elution buffer was added onto the membrane, and incubated for 5 min at room temperature (15 - 25°C) before being centrifuged for 1 min at 5,000 *g*.

Protocol 4: SureFood® PREP Advanced (R-Biopharm)

DNA was extracted following the manufacturer's protocol with some modification. Firstly, 0.15 g of sample was weighed and placed into a 2-mL microcentrifuge tube. Then, 580 µL of lysis buffer was added to the sample, and homogenised for 30 s using TissueLyser (Qiagen) for 20 s at 15 Hz. Afterward, 20 µL of proteinase K was added to the reaction tube, and mixed briefly. After incubation on a heating block (Thermo Scientific, USA) with continuous shaking for 1 h at 65°C, the sample lysate

was centrifuged (Sigma1-15, Germany) for 1 min at 12,000 rpm. Afetrwards, the liquid supernatant was transferred into a new 1.5-mL reaction tube and centrifuged again for 1 min at 12,000 rpm. The spin filter was placed into a new 2.0-mL receiver tube, and the entire supernatant from the last centrifugation step was transferred directly onto the spin filter. The spin filter was centrifuged with the receiver tube for 1 min at 12,000 rpm. After centrifugation, the spin filter was discarded. Then, 250 µL of binding buffer was added to the filtrate, and mixed well by pipetting up and down several times and vortexing. Later, the filtrate was added to the spin column tube, and incubated for 10 min at room temperature, before being centrifuged at 12,000 rpm for 1 min and placed into a new 2.0-mL receiver tube. To purify the bound nucleic acid, 550 µL of pre-wash buffer was added to the spin filter, and centrifuged at 1 min for 12,000 rpm. The filtrate was discarded, and the spin filter was placed back into the receiver tube. Afterward, 550 µL of wash buffer was added to the spin filter, and centrifuged for 1 min at 12,000 rpm. The filtrate was discarded, and the spin filter was placed back into the receiver tube. Once more, 550 µL of the wash buffer was added to the spin filter, and centrifuged for 1 min at 12,000 rpm. The filtrate was discarded, and the spin filter was placed back into the receiver tube. Subsequently, to remove the residual ethanol, the samples were centrifuged for 2 min at 12,000 rpm. Finally, to elute the DNA, the filter was placed in a new 1.5-mL receiver tube, and 50 µL of the preheated (65°C) elution buffer was added directly onto the middle bottom of the spin filter, and incubated on a heating block for 5 min at 65°C (without shaking), and finally, centrifuged for 1 min at 10,000 rpm.

Each DNA extraction protocol was used to extract 24 samples (three replicate samples of eight products). All extracts were then stored at -20°C for stable preservation.

DNA quantification and purity

DNA quantification and qualification were performed using a Nanodrop spectrophotometer (Thermo Scientific, USA). UV absorbance at 260 nm was checked to determine the DNA concentration, and the ratio of absorbance at 260 and 280 nm was noted to determine the DNA purity.

Real-time PCR using SYBR Green

The extracted DNA was then used as the template in dye-based real time qPCR using SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Master Mix (Bio-Rad, USA). PCR reactions using selected specific porcine primers (Table 2) were optimised to amplify specific expected fragments using porcine reference DNA as a template in a final volume of 20 µL. A reaction medium containing 10 µL of master mix, 1 µL of each primer at 10 µM, 1 µL of DNA template at 50 ng/µL, and 7 µL of nuclease-free water was prepared. PCR reactions with the DNA extracted from the highly processed foods were performed under the same conditions using 8 µL of DNA collected from the total eluate volume. The mixture was cycled in a CFX96 thermal cycler (Bio-Rad, USA) as follows: 3 min at 98°C, followed by 45 cycles of 10 s at 95 °C, 20 s at 59/60°C, and 10 s at 72°C. The melting curve was obtained using a temperature range starting from 65°C, with incremental increases of 0.5°C per 10 s.

Primer's specificities, sensitivities, and standard curves

Six porcine-specific primer pairs that amplify fragments smaller than 210 bp were selected from the literature (Table 2). The specificities of all included primers were optimised using the reference porcine DNA extracted from peripheral blood as a template. The PCR reactions' efficiencies were assessed by establishing standard curves, using five-fold serial dilutions of the reference porcine DNA starting from 1,000 ng. The line equation, the coefficient of determination (r^2), and the PCR efficiencies were automatically obtained by the Bio-Rad CFX Manager Software (version 3.1, Hercules, CA, USA). Only DNA pairs with an efficiency greater than 95% were included going forward. In addition, using the same optimised PCR conditions, all primers with non-specific amplifications or cross-reactions with at least one animal DNA template (chicken, cow, and turkey) were excluded from further analysis (Figure 2).

DNA purification and sequencing

To confirm the sequences of short-length fragments produced by the species-specific primers, PCR products were eluted from 2% agarose gel, purified using the Qiagen QIAquick PCR Purification Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany), and quantified using a Nanodrop instrument. Purified fragments were sequenced on Applied Biosystems 3500 Genetic Analyser using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific Inc.).

Table 2. Sequences of the selected primers for the detection of porcine-specific genes.

Primer	Target gene	Oligonucleotide sequence (5' to 3')	Amplicon size (bp)	Melting temperature (°C)	Reference
CYTOPORC	Cyt b	F: ATG AAA CAT TGG AGT CCT ACT ATT TACC R: CTA CGA GGT CTG TTC CGA TAT AAG G	149	81.5	Nikzad *et al.* (2017)
porc16SrRNA	16srRNA	F: CAACCTTGACTAGAGAGTAAAACC R: GGTATTGGGCTAGGAGTTTGTT	138	79.0	Lee *et al.* (2016)
porcmtATP8	mtATP8	F:ATCTACATGATTCATTACAATTAC R: TCATCAATAGAAACCCCCACGA	126	76.0	Yoshida *et al.* (2009) Kang *et al.* (2018)
porcD-loop	D-loop	F: TCACCACCATTAGATCACGAGC R: TCATCAATAGAAACCCCCACGA	108	85.5	Kang *et al.* (2018)
porcCytb-R1	Cyt b	F:TCCTGCCCTGAGGACAAATA R:AAGCCCCCTCAGATTCATTC	109	80.5 - 81.0	Mohamad *et al.* (2016)
porcCytb-R2	Cyt b	F:TCCTGCCCTGAGGACAAATA R: GAATAGGAGATGTACGGCTGC	203	83.5 - 84.0	Mohamad *et al.* (2016)

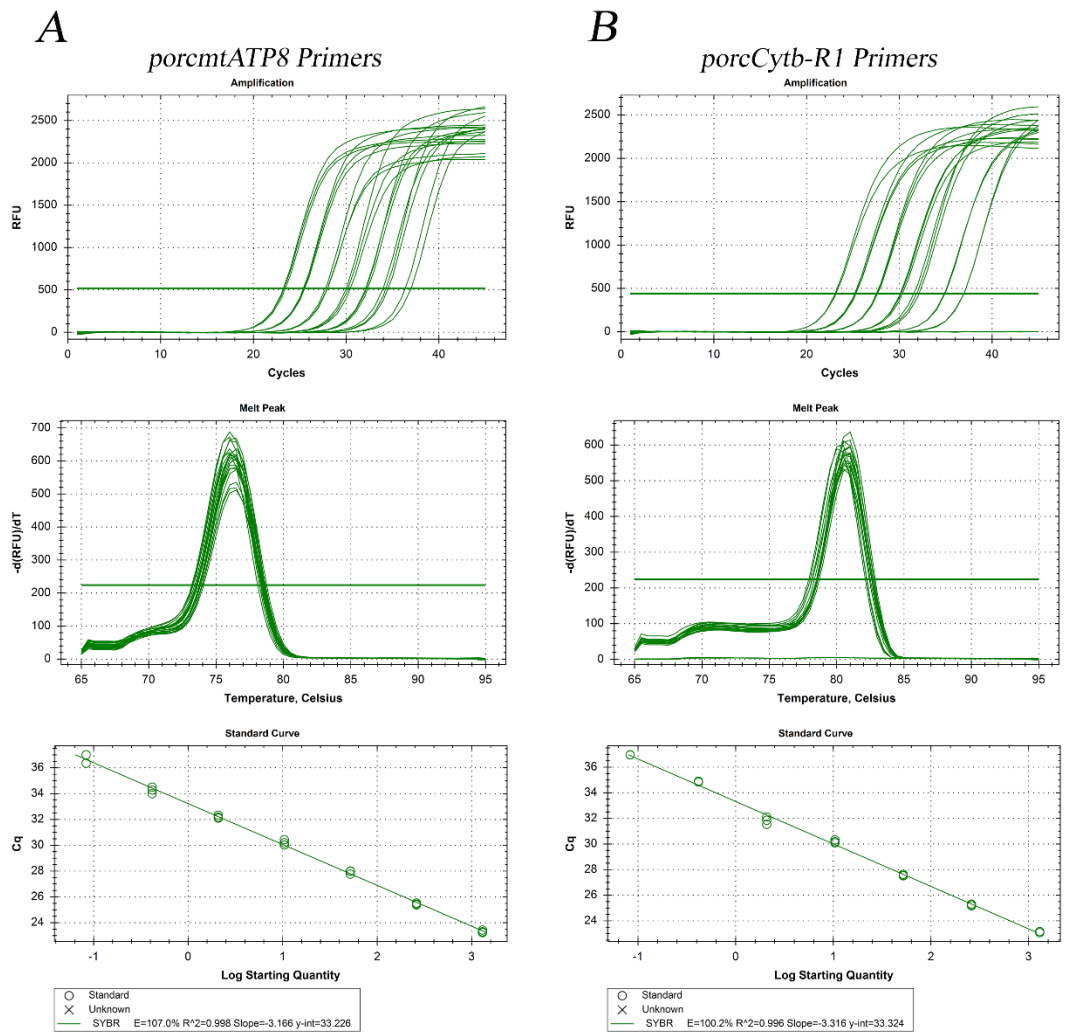

Figure 2. Quantitative PCR efficiency of ATP8 and Cytb-R1 primer pairs. **(A)** ATP8 primers: Amplification results from porcine DNA with five serial dilutions, melting curve, and standard curve line equation. **(B)** Cytb-R1 primers: Amplification results from porcine DNA with five serial dilutions, melting curve, and standard curve line equation.

Generated electropherograms were analysed using the BioEdit software. All fragments generated by the porcmtATP8 and porcCyb-R1 primers were sequenced (Figure 3).

Data analysis

The sequencing results were analysed and compared to confirm perfect matching with Sus scrofa reference sequences using Blast database (https://blast.ncbi.nlm.nih.gov).

Real-time PCR using the PowerChek™ pork gelatine and foodproof® Porcine Detection LyoKit - 5´Nuclease Kits

Commercial kits PowerChek™ Pork Gelatine (Kogenebiotech, Korea) and foodproof® Porcine Detection LyoKit (BIOTECON Diagnostics) were used to compare probe-based methods with fluorescent SYBR Green-based protocols used in the present work. The amplifications used in this real-time PCR-based protocol were carried out following the manufacturer's instructions. Reactions without DNA template (NTC) and negative controls (blanks) were used to check for DNA contamination in the PCR amplification. Amplification plots of normalised fluorescent signals versus cycles were analysed using the Bio-Rad CFX Manager Software (version 3.1, Hercules, CA, USA).

Statistical analysis

Statistical analyses were performed using the IBM SPSS version 26 software.

Figure 3. Sequencing results of the amplicons generated by dye-based quantitative PCR showing perfect matching (BLAST result) with Sus scrofa reference sequences: Using the forward primer of Cytb-R1 **(A)** and ATP8 **(B)** pairs.

Results and discussion

For religious reasons, the presence of pork derivatives in foods is not tolerable in Muslim and Judaism societies, whatever the quantities and forms are. Suppliers of food products should indicate the exact composition of the products displayed and sold to the consumers. Furthermore, by law and regulations, food producers should be able to know the composition of their products using the most sensitive molecular and chemical techniques. However, checking the authenticity of all the raw materials added to a food product is not an easy task for producers. Regardless, traces of pork derivatives are sometimes present unintentionally, due to the absence of rigorous control of the production line. Gelatine is a protein produced from the partial hydrolysis of collagen obtained from animal skin, tendons, ligaments, and/or bones. Globally, five different significant sources of gelatine have been identified, namely: porcine, bovine skin, bovine bone, fish, and poultry. In 2019, the global gelatine market demand exceeded 600 kilotons. Proportional to global meat production, most of the produced gelatine is extracted from cows or pigs. Thereby, the risk of contamination from the slaughterhouse to the consumer's dish is extremely high, even under the most stringent quality assurance measures. In the

present work, we propose a protocol for detecting traces of pork in food products to avoid false-negative results by optimising the DNA extraction conditions and minimising the cost of the test by combining SYBR Green-based methodology with commercial probe-based methods often used by accredited laboratories for such analysis.

Evaluation of DNA extraction protocols

DNA extraction is the most essential step to ensure sensitive and accurate results of unlabelled highly processed food matrix contaminated with pork gelatine. During its processing and production, gelatine is exposed to multiple extreme physical and chemical conditions. Therefore, the remaining small amount of highly degraded DNA will be the last resort to identify its origin. However, when it is added to processed foods, DNA in gelatine will be more exposed to other risks of degradation and possible mixing with additional DNA of multiple origins. DNA fragmented into small sizes is the most difficult to target, and downstream investigation must be adjusted to overcome this difficulty. Therefore, a small amount of collected DNA will reduce the chance to detect the presence of pig traces, and on the other hand, a large amount of collected DNA will increase the risk of inhibitors and/or cross-reactions (Mozayani and Noziglia, 2010). For this purpose, we selected three of the most used kits for food DNA extraction: DNeasy Mericon Food Kit (Qiagen), using two different starting sample concentrations (0.2 g standard protocol and 2 g recommended for highly processed food); foodproof® Sample Preparation Kit III (BIOTECON); and SureFood® PREP Advanced (R-Biopharm). All protocols have been optimised as described in the Materials and Methods section to maximise the recovery of pure DNA from highly processed food. Eight food products were collected from UAE and Norway markets. Their characteristics, as described by the respective suppliers, are summarised in Table 1. Twelve samples from each product were randomly selected for DNA extraction. Each collected product was extracted 12 times (thrice per protocol). A total of 96 extractions were performed (8 products, 4 protocols, 3 replicates). The collected extracts were quantified and qualified by spectrophotometer (Nanodrop, Thermo Scientific, USA). Both the concentration and 260/280 OD ratio were noted as a basic assessment of each extract. In addition, protocol efficiency was evaluated by calculating the quantity of produced DNA (ng) per g of sample.

The statistical analysis of 260/280 ratio according to the DNA extraction protocol showed a significant difference ($p = 0.005$) mainly caused by the lowest ratio obtained by foodproof® BIOTECON protocol; this might have indicated the presence of protein or another contaminant that absorbed close to 280 nm (Figure 4A). On the other hand, DNA extracted using the Qiagen kit showed the highest 260/280 ratio. However, statistical analysis based on product type as a factor showed no significant difference ($p = 0.075$) (Figure 4B). This result suggested that the variations in the 260/280 ratio were due to the protocols' processes and reagents rather than the nature of the analysed foods. Statistical analysis of the DNA extraction efficiencies (ng of DNA per gram of sample) showed a significant difference between the protocols' efficiencies ($p = 0.007$). In fact, SureFood® PREP Advanced (R-Biopharm) showed a significantly higher efficiency (Figure 4C) for all of the included products, except for the bovine gelatine powder. On the other hand, the highest efficiency was observed with the seasoned, dried pork rinds (chips) ($p < 10^{-6}$).

Most commercial PCR-based tests are designed to detect porcine DNA in processed foods, are probe-based, and use a quantitative, real-time thermal cycler. As compared to SYBR Green-based PCR, the probe-based qPCR system is chosen by most suppliers for its many advantages including higher specificity, sensitivity, reproducibility, and multiplexing options. For highly degraded DNA with a small amount of porcine material, the single-targeted genomic region by the designed primers and probe could be degraded. This could result in a false-negative decision (Figure 1). Multiplying the target regions is a solution to overcome this problem and reduce the false-negative results. However, using the probe-based method, the increase of the number of target regions could significantly inflate the cost of the single analysis. SYBR Green-based qPCR is based on the binding of a fluorescent dye to double-stranded deoxyribonucleic acid (dsDNA), while the TaqMan method uses dual dye-labelled probes associated with the exonuclease activity of the Taq polymerase enzyme; the former is more cost-effective and easier to use. Herein, we propose combining the two methods to minimise both costs and the risk of false-negative results.

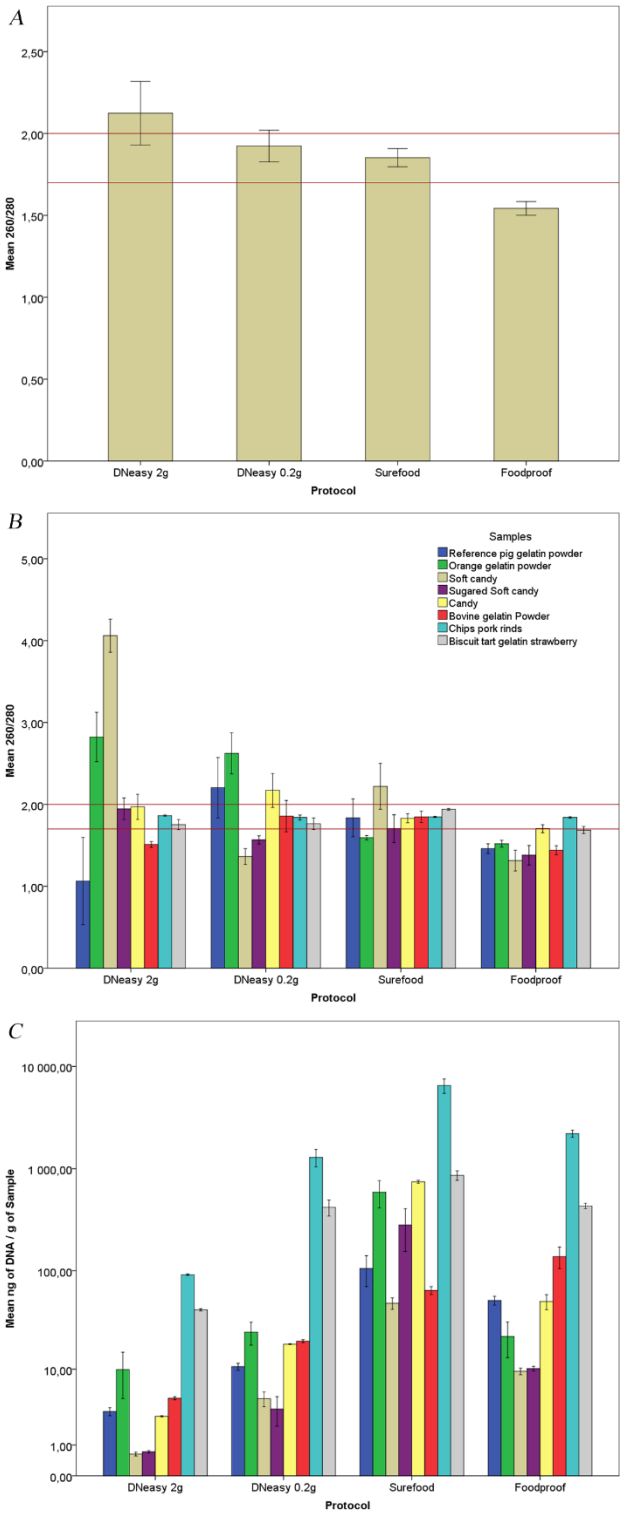

Figure 4. Evaluation of DNA extraction protocols. **(A)** Average 260/280 OD ratio according to protocol. 24 extracts were analysed for each protocol. Red lines delimit the 1.7 - 2.0 range, and error bars represent the SE. **(B)** Average 260/280 OD ratio according to the protocol (four total) and product (eight total). Three extracts per product were analysed for each protocol. Red lines delimit the 1.7 - 2.0 range. **(C)** Average DNA extraction efficiency (ng of DNA per g of sample). Three extracts per product were analysed for each protocol. Error bars represent the SE.

Evaluation of six porcine primers sets' specificity

To evaluate cross-reactions, the primers' specificity was analysed using the DNA of animal species known to often be included during slaughter, gelatine production, or food processing (*e.g.*, pig, cow, chicken, and turkey). Six mtDNA markers (D-loop region, ATP8, 3 from Cyt b region, and 16S rRNA) were assessed for their specificity for targeting porcine DNA (Table 2). Primer specificity was tested using porcine DNA extracted from peripheral blood as a template. PCR conditions were optimised based on the amplicons, and validated by a melting curve step, and finally, Sanger sequencing. Under similar amplification conditions, different templates were tested (bovine, chicken, or turkey DNA), and four pairs of primers were excluded due to non-specific amplification. The excluded primers were porcD-loop, CYTOPORC, porc16SrRNA, and porcCytb-R2 which cross-reacted, respectively, with cow and chicken, turkey, cow and chicken, cow, chicken, and turkey. Both the porcmtATP8 and porcCytb-R1 primers exhibited perfect specificities and efficiencies, and therefore, were selected for screening samples in combination with the commercial probe-based primer pairs.

Efficiency and sensitivity of the quantitative PCR reaction

To assess the efficiency of the quantitative PCR system of the retained sets of porcine-specific primers mtATP8 and cytb-R1, standard curves were generated using 10 serially diluted samples of porcine DNA starting from 1 µg (5-fold serial dilution: 1,000, 200, 40, 8, 1.6, 0.32, 0.064, 0.0128, 0.0256, and 0.000512 ng). The PCR was optimised for efficiencies close to 100%, following the criteria of the Codex Alimentarius (Codex, 2010) (Figure 2).

Sample screening

One hundred twenty-eight runs were performed using the ATP8 and Cytb-R1 primer sets (64 for each). Using the four optimised protocols, all product extracts were tested in duplicate for porcine DNA detection (8 samples × 4 protocols × 2 = 64). In addition, for each sample, the highest C_t value plus a random selection of negative results obtained and C_t values outside of the dynamic range for both ATP8 or Cytb-R1 primers were analysed with both commercial kits (45 runs were performed, an average of 5 runs for each product) to evaluate false-positive and false-negative results. A statistical comparison of

the C_t means obtained with the ATP8 and Cytb-R1 primers showed no significant differences ($p > 0.05$). However, a significant difference was observed for the different protocols ($p = 0.00001$). In fact, the extracts obtained with foodproof® Extraction Kit (BIOTECON) showed negative results for four different products including orange gelatine powder, soft candy, sugared soft candy, and bovine gelatine (Table 3). This result is in concordance with the evaluation from the extraction protocol showing a significantly lower 260/280 ratio for the extracts obtained using the foodproof® Extraction Kit. Analysis of the distribution of the positive/negative results following the extraction protocols showed a significant difference ($p = 0.019$) caused by the positive results obtained using the SureFood (23 *vs* 9) and DNeasy 0.2 g (21 *vs* 11) extraction protocols ($p = 0.0025$). On the other hand, the high number of positive reactions obtained with the Cytb-R1 set of primers when compared with ATP8 (40/64 *vs* 31/64) could be explained by the smaller size of the target amplicon (109 *vs* 126 bp). This finding emphasised the importance of target sequence multiplicity in detecting porcine traces in a highly processed sample. Two samples expected to be positive for porcine DNA (reference gelatine powder and chips) generated 100% of positive results (Table 3) using both the in-house and commercial protocols.

Table 3. Amplification results according to extraction protocol, primers, and commercial kit.

		Sample								Total
		Reference	**Orange gelatine powder**	**Soft candy**	**Sugared soft candy**	**Candy**	**Bovine gelatine powder**	**Chips**	**Biscuits**	
Protocol										
DNeasy 0.2 g	Positive[1]	4	2	1	1	3	2	4	4	21
	Negative	0	2	3	3	1	2	0	0	11
	Total	4	4	4	4	4	4	4	4	32
DNeasy 2 g	Positive	4	1	1	2	1	1	4	1	15
	Negative	0	3	3	2	3	3	0	3	17
	Total	4	4	4	4	4	4	4	4	32
foodproof	Positive	4	0	0	0	3	0	4	1	12
	Negative	0	4	4	4	1	4	0	3	20
	Total	4	4	4	4	4	4	4	4	32
SureFood	Positive	4	4	2	3	4	1	4	1	23
	Negative	0	0	2	1	0	3	0	3	9
	Total	4	4	4	4	4	4	4	4	32
Primer										
ATP8[#]	Positive	8	2	2	2	4	1	8	4	31
	Negative	0	6	6	6	4	7	0	4	33
	Total	8	8	8	8	8	8	8	8	64
Cytb-R1[2]	Positive	8	5	2	4	7	3	8	3	40
	Negative	0	3	6	4	1	5	0	5	24
	Total	8	8	8	8	8	8	8	8	64
Total										
	Positive	16	7	4	6	11	4	16	7	71
	Negative	0	9	12	10	5	12	0	9	57
	Total	16	16	16	16	16	16	16	16	128
PCR Commercial kit										
Powercheck[3]		+ (3/3)	+ (2/4)	- (0/3)	- (0/2)	+/- (1/4)	- (0/3)	+ (3/3)[5]	- (0/2)	9/24
foodproof[4]		+ (2/2)	+ (2/4)	+ (2/3)	+ (1/2)	+ (1/2)	+ (2/3)	+ (3/3)[5]	- (0/2)	13/21
Expected results		+	+	Unknown	Unknown	+	-	+	+	

[1]A run is considered positive for a primer only when the Ct value falls within the dynamic range of the standard curve; [2]8 µL of extract as the template; [3]5 µL of extract as the template; [4]25 µL of extract as the template; and [5]positive results obtained even at 10× and 100× dilutions.

However, the remaining samples that according to their manufacturers contained porcine derivatives (orange gelatine powder, candy, and biscuits) showed variable percentages of positive reactions using the Cytb-R1 or ATP8 sets of primers, ranging from 44% (7 of 16 runs) to 69% (11 of 16 runs). Surprisingly, although labelled as containing porcine ingredients, all of the reactions performed with the commercial kits showed negative results with biscuits (4 of 4 runs; 100%). However, using the in-house SYBR Green-based protocol, 7 of 16 runs (43.7%) were positive, which highlighted the importance of both sample and target multiplicity. In contrast, two samples of soft candies purchased from a Norway market were tested as unknown samples. The results showed variable positive reactions using the Cytb-R1 and ATP8 sets of primers, ranging from 33.3% (4 of 12 runs) to 60% (6 of 10 runs). Several factors may influence the risk of false-negative results starting with the efficiency of DNA extraction, which differs case by case depending on the processing procedure. Moreover, downstream processes can be affected by the presence of inhibitors such as residual polysaccharides, proteins, food additives (*e.g.*, preservatives, emulsifiers, antioxidants, and stabilisers), or unknown contaminants. Consequently, the abundance of ingredients (non-targets) in several food categories and the low amount of target DNA in the samples increases the risk of false-negative results.

In addition, the bovine gelatine powder purchased from a UAE market and expected to be free of any traces of pig derivatives had 25% positive reactions (4 of 16). Three positive reactions out of four were obtained using the Cytb-R1 primer pair, and confirmed by directly sequencing the amplicons. This positive result was reproduced using the foodproof® Rt commercial kit. In contrast, all reactions performed using the PowerChek™ Kit were negative. The discrepancy between the results from the commercial kits could be explained by the concurrency of several factors including the sizes of the sequences targeted by the different probes, the chemical composition of the reaction medium of each commercial kit, the behaviour of each kit with the PCR inhibitors, and the sensitivity of each kit to the low porcine proportion in the used extracts. This last reason seemed the most plausible explanation in our case. In fact, following the manufacturer's instructions, 25 µL of DNA extract was used to carry out a reaction with the foodproof® Porcine Detection Kit, while only 5 µL was used in the case of the PowerChek™ Kit.

Conclusion

The present work highlighted the importance of targeting several small regions of the mitochondrial genome to effectively detect small traces of porcine products. It is also recommended to use the DNeasy and SureFood kits for optimal DNA extraction. The combination of SYBR Green (Cytb-R1) and probe-based methods (foodproof) optimised running costs and reduced the risk of false-negative conclusions. These findings could help food control laboratories to ensure that food products (*e.g.*, gelatine) comply with *halal* regulations in Muslim local markets.

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