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Detection of porcine DNA in cooked meatballs using polymerase chain reaction (PCR) assay

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Abstract

This study was conducted to detect the presence of porcine DNA in meatballs using polymerase chain (PCR) reaction assay targeted mitochondrial DNA (mtDNA) species-specific gene. Meatballs spiked with 1.0% (w/w) and 5.0% (w/w) porcine meat and gelatin, respectively, were prepared and heat-treated using five (n=5) cooking methods: boiling, pan-frying, roasting, microwaving and autoclaving. Two pairs of mtDNA primers were targeted in short sequences using polymerase chain reaction (PCR) analysis, producing 212- and 83-bp amplicons. Electrophoresis analysis showed positive results for porcine DNA at 1.0% and 5.0% for both porcine meat and gelatin for all of the different cooking techniques. Thus, PCR analysis using species-specific primers was demonstrated to be very useful for the detection of porcine DNA in cooked meatballs.

Introduction

Meatballs are produced from a mixture of finely ground meat, prepared using beef, chicken, fish or porcine (Arief *et al*., 2012). Different meatball products are popular across various cultures throughout the world. In Malaysia, meatballs are typically prepared using beef and are referred to as bebola daging. The beef used in meatballs is considered to be premium meat and there is a tendency to mix beef with lower value meat, such as porcine (Aida *et al*., 2005). Beside the fraudulent of meat, the food ingredients such as porcine gelatin is also possibly added into meatballs to improve the texture, water holding capacity and the juiciness of meatballs (Aravindran *et al.*, 2014).

Porcine meat and porcine-derivatives such as porcine gelatin are non-halal and not allowed to be consumed under Shariah laws. The issue of using gelatin is alarming and sometimes controversial due to commercial gelatins are exclusively porcine-based since the emergence of Bovine Spongiform Encephalopathy (BSE) or mad cow disease in the 1980s, which restricted the use of bovine gelatin (Morrison *et al.*, 1999). Furthermore, porcine gelatin is cheaper compared to bovine gelatin due to shorter production time of porcine gelatin, it takes about 30 days, while bovine gelatin production lasted between 60-80 days, thus this affect the product cost. The halal gelatin from bovine source must fulfill the Shariah requirement such as; the bovine must be slaughtered by a Muslim and processed according to Shariah Laws (Sahilah *et al*., 2015). The alternative gelatin sources are fishes (fish gelatin), seaweeds (carrageenan) and gum Arabic but these could not fulfil various industries demand. Sahilah *et al*. (2015) reported that the various use of porcine gelatin in industries is expanding and the exposure of haram (non-halal) gelatin is not only towards Muslims but also other communities such as Jews, vegetarians and a number of people who are allergic toward hidden porcine ingredients and meat sources in processed foods.

The most frequent approach used to determine the presence of porcine DNA in food materials and to identify questionable food product ingredients is to use the DNA amplification of specific target genes from mitochondria DNA (mtDNA) (Matsunaga *et al.*, 1999; Sahilah *et al.*, 2011). mtDNA-based methods are considered to be more reliable because the DNA is stable and resistant under the high temperature, high pressure and chemical treatment conditions that are used in food processing in which other types of DNA have typically been degraded (Madesis *et al*.,

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2014).

In the present study, we aim to detect porcine DNA by spiking porcine meat and gelatin in meatballs and treating them with five different cooking methods, specifically boiling, pan-frying, roasting, microwaving and autoclaving, using mtDNA for the transfer of RNA-ATP8 (tRNA-ATP8) (Tartaglia and Saulle, 1998; Lahiff *et al*., 2001) and ATP6 primers (Yoshida *et al*., 2009). The treatment and different types of meatballs prepared represent the DNA quality of commercial meatball products after heat processing and, in turn, assists in porcine DNA detection in any possible heat-treated samples collected in marketplaces.

Materials and Methods

Meatball preparation

A total of four (4) types of meatballs with a different percentage of porcine meat and gelatin were prepared as indicated in Table 1.

Meatballs were made according to basic formulations, according to Azhana (2011), with some modification. First, meats (minced beef and porcine meat or pork) were mixed with shortening and ice cubes for 1 mins. Then, dried ingredients, including soy protein isolate (ISP), sodium triphosphate (STPP), potato starch flour, black pepper powder, salt and sugar, were weighed and blended together for another 1–2 mins to form homogeneous dough. The dough was then stored in a refrigerator for 20 mins before being shaped into a ball with a weight of approximately 10g each. The meatballs were then boiled at 90–95°C for 3–5 mins, then soaked in ice water for 10 mins and finally drained to dry. Finally, all of the meatballs were packed in Ziploc® plastic bags and stored in a freezer at -20°C until used. The same steps were repeated by replacing the porcine meat with porcine gelatin.

Meatballs spiked with 1.0% (w/w) and 5.0% (w/w) porcine meat and gelatin were further treated with five (5) different cooking methods; boiling water with 2.0% (w/v) of salt at 90-95°C for 5 mins; pan-frying with vegetable oil for 5 mins; roasting in oven at 180°C for 5 minutes; microwaving at medium level for 5 mins; and autoclaving at 121°C using temperature-resistant container added with 250 ml of hot water and 2.0 (w/v) of salt for 20 mins. All cooking methods were based on Arslan *et al.* (2006) with a slight modification in which the temperature and time were adjusted.

DNA extraction

All of the meatballs were minced, and a total of 100 mg of each type were transferred into a 2.0 ml sterile microcentrifuge tube. The DNA was extracted using a Qiagen D'Neasy Mericon Food Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with some changes, and then eluted with 50 µl of an EB buffer and quantified using a MaestroNano® Spectrophotometer (MaestroGen, Nivada, USA). The DNA was then stored at -20°C until further analysis. All of the DNA of the meatball samples was extracted in duplicate from each source.

Table 1. Basic ingredients for meatball which added with meat or porcine gelatin.

Ingredients	Percentages of porcine meat or gelatin	
	1.0% (w/w)	5.0% (w/w)
Minced meat	69.0	65.0
Porcine meat/gelatin	1.0	5.0
Shortening	5.0	5.0
Isolate soy protein (ISP)	4.5	4.5
Sodium triphosphate (STPP)	0.3	0.3
Potato starch flour	3.6	3.6
Black pepper	0.1	0.1
Salt	1.5	1.5
Sugar	2.0	2.0
Ice cubes	13.0	13.0

Oligonucleotide primers

The oligonucleotide primers targeting mitochondrial DNA (mtDNA) regions of the transfer RNA-ATP8 (tRNA-ATP8) (Tartaglia and Saulle, 1998; Lahiff *et al.*, 2001) and ATP6 primers (Yoshida *et al*., 2009) were used in the PCR assays. The sequences of those primers were tRNA-ATP8 (F), 5'-GCC TAA ATC TCC CCT CAA TGG TA-3' and tRNA-ATP8 (R), 5'-ATG AAA GAG GCA AAT AGA TTT TCG-3'); and ATP6 (F), 5'-CTA CCT ATT GTC ACC TTA GTT-3' and ATP6 (R), 5'-GAG ATT GTG CGG TTA TTA ATG-3'). All of the mtDNA primers were synthesized and supplied by First Base Laboratories Sdn. Bhd. (Selangor, Klang, MY).

PCR amplification

The PCR amplification technique using the tRNA-ATP8 primers targeting mtDNA of 212 bp (Tartaglia and Saulle, 1998; Lahiff *et al*., 2001) was performed at a final volume of 50 µl containing 25 µl of DreamTaq Green PCR Master Mix (2X) (Fermentas, Vilnius, Lithuania), 1 µl of 5 µM for each primer (forward and reverse), 21 µl of nuclease free water (NFW) and 2 µl

of an approximately 100-ng DNA template. Negative and positive DNA controls were prepared by adding 2 µl of NFW and Pig Genomic DNA (Novagen®, Darmstadt, Germany), respectively. A mastercycler® gradient thermal cycler (Eppendorf, USA) was used to run the PCR with a temperature program consisting of an initial denaturation at 95°C for 2 mins, followed by 30 cycles of heating at 94°C for 1 mins, 55°C for 1 mins, 72°C for 2 mins and a final extension step at 72°C for 10 mins. The amplification products were electrophoresed through a 2.5% (w/v) agarose gel in a 1 X TAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 8.0) at 100V for 40 mins and pre-stained with MaestrosafeTM Nucleic Acid (V-BioScience, Kuala Lumpur, MY).

The porcine DNA was amplified using an 83-bp target primer of ATP6 (Yoshida *et al*., 2009) in a 50 µl reaction volume containing 25 µl of DreamTaq Green PCR Master Mix (2X) (Fermentas, Vilnius, Lithuania), 1 µl of 5 µM for each primer (forward and reverse), 21 µl of nuclease free water (NFW) and 2 µl of an approximately 100-ng DNA template. Negative and positive DNA controls were performed as mentioned above. PCR was also performed in a Mastercycler® gradient thermal cycler (Eppendorf, Hamburg, Germany) with a temperature program consisting of the initial heat activation at 95°C for 9 mins, followed by 45 cycles of 92°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and a final extension step at 72°C for 5 mins. The PCR products were separated by electrophoresis through a 3% (w/v) agarose gel in 1X TAE buffer (40mM Tris-acetate, 1mM EDTA, pH 8.0) at 100V for 45 mins and pre-stained with MaestrosafeTM Nucleic Acid (V-BioScience, Kuala Lumpur, MY).

All of the agarose gel electrophoreses of the PCR product used a GeneRulerTM 100-bp DNA ladder (Fermentas, Vilnius, Lithuania) as a molecular size marker and were visualized using a UV Gel Documentation System (Syngene, Cambridge, UK).

Detection limit of oligonucleotide primers

The detection limit of all of the oligonucleotides described above was cross-examined using Pig Genomic DNA (Novagen®, Darmstadt, Germany). The PCR assay condition was similar to that described in the PCR amplification using different oligonucleotide primers with a different concentration of porcine DNA ranging from 0 to 150ng.

Results and Discussion

In the present study, we prepared 1.0% (w/w) and 5.0% (w/w) porcine meat and gelatin meatballs, respectively, and treated them with five different cooking methods, including boiling, pan-frying, roasting, microwaving and autoclaving.

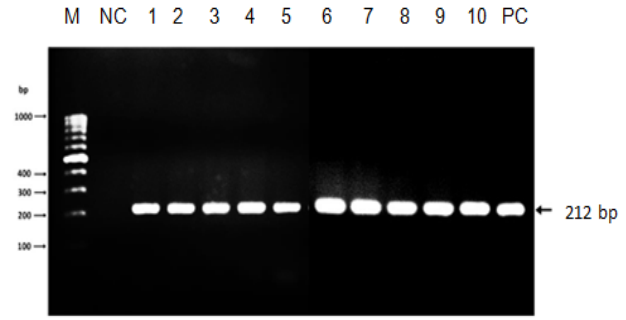

Figure 1. PCR amplification product of porcine meatballs using tRNA-ATP8 primers on 2.5% (w/v) agarose gel with different percentage of porcine meat. Lane 1 to 5 for 1.0% (w/w) concentration of porcine meat; Lane 6-10 for 5.0% (w/w) concentration of porcine meat. M, 100bp DNA ladder; NC, negative control; lane 1 and 6, boiling; lane 2 and 7, pan frying; lane 3 and 8, roasting; lane 4 and 9, microwaving; lane 5 and 10, autoclaving; PC, positive control (212 bp).

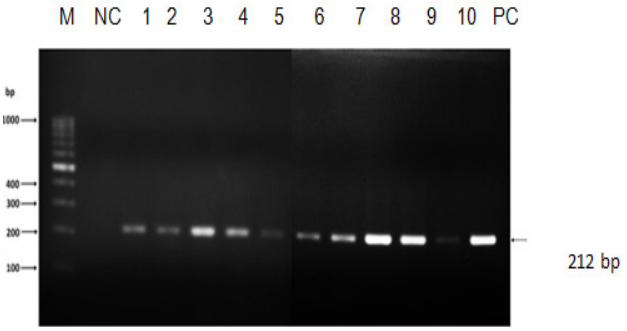

Figure 2. PCR amplification product of porcine gelatin meatballs using tRNA-ATP8 primers on 2.5% (w/v) agarose gel with different percentage of porcine gelatin. Lane 1 to 5 for 1.0% (w/w) concentration of porcine meat; Lane 7-10 for 5.0% (w/w) concentration of porcine meat. M, 100bp DNA ladder; NC, negative control; lane 1 and 6, boiling; lane 2 and 7, pan frying; lane 3 and 8, roasting; lane 4 and 9, microwaving; lane 5 and 10, autoclaving; PC, positive control (212 bp).

The tRNA-ATP8 and ATP6 primers were selected to detect porcine DNA in heat-processed meatballs due to their ability to detect porcine DNA in feed samples that had undergone heat and denaturing treatment during the manufacturing process (Tartaglia and Saulle, 1998; Lahiff *et al.*, 2001; Yoshida *et al.*, 2009). A similar assumption was applied to the meatballs, which also underwent heat treatment during the industrial-scale manufacturing process. The tRNA-ATP8 primer, developed by Lahiff *et al.* (2001), targeted the mtDNA of transfer RNA for lysine (tRNALys) and the ATPase8 gene (GenBank database Accession no. AF039170); these

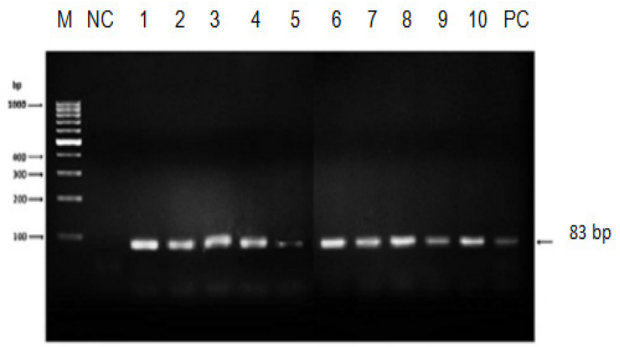

Figure 3. PCR amplification product of porcine meatballs using ATP6 primers on 3.0% (w/v) agarose gel with different percentage of porcine meat. Lane 1 to 5 for 1.0% (w/w) concentration of porcine meat; Lane 7-10 for 5.0% (w/w) concentration of porcine meat. M, 100bp DNA ladder; NC, negative control; lane 1 and 6, boiling; lane 2 and 7, pan frying; lane 3 and 8, roasting; lane 4 and 9, microwaving; lane 5 and 10, autoclaving; PC, positive control (83 bp).

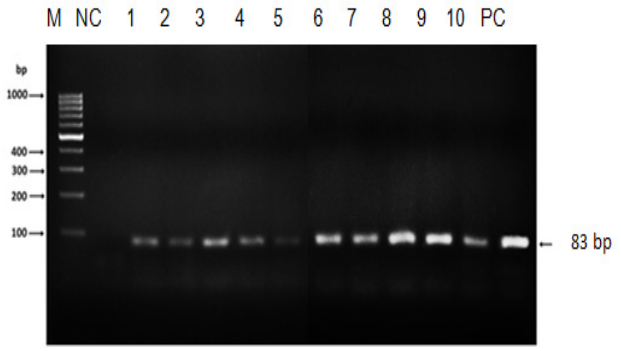

Figure 4. PCR amplification product of gelatin meatballs using ATP6 primers on 3.0% (w/v) agarose gel with different percentage of porcine gelatin. Lane 1 to 5 for 1.0% (w/w) concentration of porcine meat; Lane 7-11 for 5.0% (w/w) concentration of porcine meat. M, 100bp DNA ladder; NC, negative control; lane 1 and 7, boiling; lane 2 and 8, pan frying; lane 3 and 9, roasting; lane 4 and 10, microwaving; lane 5 and 11, autoclaving; PC, positive control (83 bp).

are flanked by cytochrome oxidase II (COII) at the 5–end and the ATPase6 gene at the 3 end (Tartaglia and Saulle, 1998), whereas the ATP6 primer was encoded at the ATPase6 gene in mtDNA (Yoshida *et al*., 2009). Figure 1 and Figure 2 show a different intensity of bands among meat and gelatin porcine meatballs with the tRNA-ATP8 primers, as observed on an agarose gel. Specific bands of 212bp observed on the gel were present at a high intensity for porcine meatballs (Figure 1) compared to porcine gelatin meatballs (Figure 2). This result may indicate that the DNA extracted from porcine fresh meat was less degraded compared to the DNA associated with porcine gelatin. Gelatin is a protein from animal collagen that undergoes several harsh processing steps during manufacturing, and the process leads to the denaturation of the associated DNA.

Although all of the meatball samples were cooked as shown Figure 1 and Figure 2, the porcine DNA was still detected in both the 1 and 5% (w/w) quantities. A low intensity of a specific band was easily observed on the agarose gel for porcine gelatin meatballs. Figure 2 showed autoclaved porcine gelatin meatballs exhibited the lowest intensity band on the agarose gel compared to the other bands. This result was consistent with that of Meyer *et al*. (1994) who reported that food processing at high temperatures could lead to DNA degradation, thus affecting its detection. Pascoal *et al*. (2005) also reported a similar finding that indicated that although DNA exhibits fairly high thermal stability, the intense heat coupled with high-pressure conditions may cause severe DNA damage and affect the quality of the recovered DNA. That may explain why the DNA band of autoclaved meatballs was faded (Figure 2). The respective DNA in autoclaved meatballs still provides sufficient target sequences to enable identification (Bellagamba *et al*., 2001). This result was also supported by Teletchea *et al.* (2005) who reported that despite the DNA being degraded and altered, it was still possible to amplify small DNA fragments, allowing for species identification.

The ATP6 was subsequently used for detecting porcine DNA in meatballs due to the assumption that small amplicons produced after PCR analysis were desirable to maximize the chance of obtaining positive results from samples that had been harshly heat-treated (Pascoal *et al.*, 2005; Arslan *et al.*, 2006). The ATP6 primer produced 83-bp amplicons, which were two and a half times smaller than those produced by the tRNA-ATP8 primer (212 bp). As shown in Figure 3 and Figure 4, specific bands of 83 bp on an agarose gel were observed on neither porcine nor gelatin meatballs. This result was expected due to the ability of the ATP6 primer to detect fragmented or damaged porcine DNA that had been heat-treated (Yoshida *et al*., 2009). Thus, both primers, tRNA-ATP8 and ATP6, were useful for porcine DNA detection in cooked meatballs.

The detection limit of primers tRNA-ATP8 and ATP6 for detecting a target sequence was 0.1 ng (tRNA-ATP8) and 0.0001 ng (ATP6), respectively (Corona *et al*., 2007; Yoshida *et al*., 2009). Our detection limit results were consistent with the findings stated by Corona *et al.* (2007) and Yoshida *et al.* (2009), who reported similar values of a genomic DNA detection limit. The detection limit of the ATP6 primer showed a higher sensitivity to detect porcine DNA compared to the tRNA-ATP8 primer. Our finding was in agreement with Yoshida

et al. (2009) who reported the primer's ability to detect porcine DNA in meat and bone meal (MBM); they demonstrated that the ATP6 primer has high specificity and sensitivity for detecting porcine DNA in various rendering procedures in Japan.

The specificity of primer tRNA-ATP8 and ATP6 have been conducted by Corona *et al* (2007) and Yoshida *et al.* (2009), respectively showed that those primers were species-specific for porcine DNA. No band was shown for cattle, sheep, goat, horse, deer, rabbit, mouse, rat, human, whale chick, quail and duck for ATPs primers. While primers tRNA-ATP8 did not amplified cattle, sheep, goat and chicken (Corona *et al.*, 2007). We also analyzed similar animals meat using tRNA-ATP8 primers the data showed (data not published) the same result thus this primer was species-specific for porcine. Therefore, no further sequence analysis was conducted in this study since those primers were porcine species-specific. We did not use polymerase-restriction fragment length polymorphism (PCR-RLFP) analysis as reported by other researchers due to our attention to detect the porcine DNA using species-specific primers (Wolf *et al.*, 2000; Chandrika *et al.*, 2009; Sahilah *et al*., 2012). Furthermore, PCR technique is faster compared to PCR-RFLP analysis.

Conclusion

This study demonstrated that mitochondrial (mt) DNA amplified by the tRNA-ATP8 and ATP6 primers was useful for detecting porcine DNA in meat and gelatin meatball products.

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