



Original article

Species-specific identification of porcine blood plasma in heat-treated chicken meatballs



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ABSTRACT

This study was conducted to detect the presence of chicken and porcine DNA in meatballs using mitochondria DNA (mtDNA) of cytochrome *b* (cyt *b*) and nuclear DNA (nDNA) short interspersed nuclear element (SINE) species-specific primers, respectively. While, the mtDNA primers targeted transfer RNA-ATP8 (tRNA-ATP8) gene was used for 1 and 5% (w/w) chicken meatball spiked with commercial porcine blood plasma. Chicken meatballs spiked with 1% and 5% (v/w) fresh and commercial porcine blood plasma, respectively were prepared and heat-treated using five ($n = 5$) cooking methods: boiling, pan-frying, roasting, microwaving and autoclaving. Two pairs of mtDNA and nDNA primers used, produced 129 and 161 bp amplicons, respectively. Whereas, tRNA-ATP8 primers produced 212 bp of amplicon. Electrophoresis analysis showed positive results for porcine DNA at 1% and 5% (w/w or v/v) for all of the different cooking techniques, either for fresh or commercial blood plasma using SINE primers but not for tRNA-ATP8 primers. The present study has highlighted the useful of species-specific primers of SINE primers in PCR analysis for detecting porcine DNA blood plasma in heat-treated chicken meatballs.

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1. Introduction

The blood is a red fluid containing water, cells, enzymes, protein and other organic and inorganics compounds. Plasma represents 60% (by volume) of entire blood and 30–40% are cellular fraction disperses in liquid fraction. After removing the cells from unclotted blood, blood plasma contents 6–8% proteins consisting primarily of albumin, globulins and fibrinogen (Bah et al., 2013). Blood is the by-product of meat industry and the discharge of blood in high volume in environment is avoidance due to increase the biochemical oxygen demand (BOD) in the water source and kills other organism such as fish which produce undesirable smell and can cause air pollu-

tion. The other alternative is to recycle the blood to obtain different forms of proteins such as blood plasma, enzymes and extraction of bioactive peptides (Bah et al., 2013). The blood-derivatives form valuable protein additives due to their functional and nutritional properties (Donnelly et al., 1978; Del Rio et al., 1980). One of that is blood plasma which is yellow powder and containing about 70% protein (Donnelly et al., 1978). Porcine plasma are containing 8.5% total solid, 1.7% of ash, 6.1% of protein, 0.2% of non-protein nitrogen and 0.2% of fat with the mean pH value of 7.8 (Donnelly et al., 1978). While, chicken plasma content 79.6% of crude protein for the dialysed procedure, low content of ash (4.60%), carbohydrates (14.99%) and non-protein nitrogen (0.75%) (Del Rio et al., 1980).

Due to tremendous growth of quality processed-product in the market, blood plasma is possible to be added in any meatball due to its ability serves as emulsifier, solubility and foaming capacity thus increase the elasticity of the meatball product. Blood plasma is also can be substituted the function of egg albumin, soy bean and milk protein in bakery products (Dill and Ladmann, 1975). While, spray-dried bovine and porcine plasma were added as feed animal to enhance the growth rate and feed intact of the animal (Bah et al., 2013).

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Blood is prohibited for Muslim consumers either it comes from halal (legal and permissible) or non-halal (illegal or impermissible) animal. Any foods added with blood are non-halal according to *Shariah* law it includes the blood-derivatives such as plasma protein and transglutaminase (TGase). In Malaysia, *Shariah* law based on Al-Qur'an, the Sunnah, consensus of opinions of Muslims jurists (*ijma'*) and analogy (*qiyas*) and other modes of legal reasoning (*ijtihad*). *Shariah* rulings based on *ijtihad* or '*fatwa*' on Halal matters should be in line with the Shafie *Mazhab of Ahlul-Sunnah wa al-Jamaah* (Sahilah et al., 2016). This is in line with Malaysian Standard MS1500:2019 Halal Food General Requirement (Third revision), state that the halal food does not consist of or contains any part of matter of an animal prohibited by *Shariah* law and *fatwa*. Since the blood plasma protein is come from the extraction of the animal blood, the blood plasma is considered as non-halal to be consumed. The presence of any animal component or part in food might concern Jews, vegetarian and a number of people who allergic towards porcine in the processed food (Tanabe et al., 2007; Sahilah et al., 2015)

Concerning on the Halal status the DNA-based PCR analysis is the most reliable method to detect porcine DNA in food samples. The DNA-based PCR analysis is the method of choice due to its sensitivity and reproducibility (Tanabe et al., 2007). Several researchers have used the PCR-based analysis were Cheng et al. (2003), Corona et al. (2007), Yoshida et al (2009), Laila Liyana et al. (2018) and Safiyah et al. (2020). Among frequent target sequence used for animal species detection is mitochondrial DNA (mtDNA) of cytochrome *b* gene, transfer RNA-ATP8, ATP6, ATP8 and others (Laila Liyana et al., 2018). The mtDNA is a sensitive marker, stable and resistant under the high temperature and chemical treatment (Koyishi et al., 2002). While, for short interspersed repetitive elements (SINE) sequences in genome is also reported as sensitive as mtDNA markers for animal species identification (Calvo et al., 2001; Koyishi et al., 2002). Calvo et al. (2001) have amplified 55 pig blood samples, raw meat, fat and other processed products such as hamburger and sausage. The rapid and sensitive techniques are useful to determine the halal status of the food products in avoiding mislabeling or fraudulent substitution. In this study, we used mtDNA of *cyt b* and SINEs primers to detect chicken and porcine DNA, respectively on chicken meatballs heat-treated using five ($n = 5$) cooking methods: boiling, pan-frying, roasting, microwaving and autoclaving.

2. Materials and methods

2.1. Samples for analysis

Different types of meatballs were prepared and spiked with 1% (v/w) and 5% (v/w) fresh and commercially available blood plasma (Sigma-Aldrich, MY), respectively. A total of 40 (20*2) (fresh porcine blood plasma and commercial) chicken's meatball were made and stored at 4 °C prior to PCR analysis. A commercial Pig Genomic DNA (Novagen®, Germany) and extracted chicken's DNA from chicken meat were used as a positive control.

2.2. Meatball preparation

A total of two (2) types of meatballs with a different percentage of porcine blood plasma were prepared according to Azhana (2011) formulation as listed in Table 1. The meatballs were boiled at 90–95 °C for 3–5 min, then soaked in ice water for 10 min and finally drained to dry. All meatballs were packed in Ziploc® plastic bags and stored in a freezer at –20 °C prior to use. The same steps were repeated by replacing the fresh porcine blood plasma with commercial porcine blood plasma.

Table 1
Basic ingredients for meatball which added with chicken meat or blood plasm.

Ingredients	Percentages porcine blood plasm	
	1.0% (w/w)	5.0% (w/w)
Chicken minced meat	69.0	65.0
Porcine blood plasm (v/w in liquid form; w/w in powder form)	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

Meatballs spiked with 1% and 5% (w/w) porcine blood plasm (fresh and commercial) were further treated with five (5) different cooking methods – boiling, pan-frying, roasting, microwaving and autoclaving – based on a study by Arslan et al. (2005), with a slight modification in which the temperature and time were adjusted. Table 2 summarises the methods for each treatment.

2.3. Preparation of fresh porcine blood plasma

The porcine (*Sus scrofa*) blood sample was obtained from local market in Muar, Johor on May 2015. All pig has been certified health by local Department of Veterinary Muar before slaughtered. The fresh blood was collected in 15 ml centrifuge tubes and placed in ice at 4 °C prior analysis.

The blood was centrifuged using Rotofix 32A Benchtop Centrifuge (Hettich Centrifuges UK) for 15 min at 2000–2500 rpm (Williams et al., 2014). Two layers were formed, the upper was blood plasma with yellow color, while the lowest was red blood cells with red color (Stefan et al., 2006). The upper layer was taken and stored at –20 °C until use.

2.4. DNA extraction

A total of 300 mg minced meatball of each type was transferred into a 2.0-ml sterile micro centrifuge tube and DNA was extracted using a Qiagen Blood and Tissue Kit (Germany) according to the manufacturer's instructions. The DNA was eluted with 50 µl of an AE buffer and quantified using a MaestroNano® Spectrophotometer (MaestroGen, USA). The DNA was then stored at –20 °C until further analysis. All of the DNA of the meatball samples were extracted in duplicate from each source.

2.5. Oligonucleotide primers

The oligonucleotide primers targeting mitochondrial DNA (mtDNA) regions of cytochrome *b* (Aravindran et al., 2015) for

Table 2
Method used to cook meatballs.

Heat treatments	Condition	Temperature (°C)	Time (min)
Boiling	Boiling water with 2.0% (w/v) of salt	90–95	5
Pan frying	Vegetable oil	–	5
Roasting	Conventional oven	180	15
Microwaving	Medium level	–	5
Autoclaving	Temperature-resistant container, containing 250 ml of hot water and 2.0% (w/v) of salt	121	20

– Couldn't determine the temperature.

chicken and oligonucleotide primers targeting nucleus DNA (nDNA) regions of short interspersed nucleus element (SINE) (Calvo et al., 2001) were used in the PCR assays. The sequences of those primers were cyt b (F), 5'-5'- CCT AAC TTG ATT CAC CTT CTC TCT GC-3' and cyt b (R), 5'- GAA GCT TAG GTT CAT GGT CAG GT-3' (chicken); and SINE (F), 5' - GGA TCC GGC ATT GCC GTT AG- 3'and SINE (R), 5' - GTC TTT TTT TGC CAT TTC TTG G-3' (porcine). While, for transfer RNA-ATP8 primers (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') (porcine) (Lahiff et al., 2001). All of the mtDNA and nDNA primers were synthesized and supplied by First Base Laboratories Sdn. Bhd. (Selangor, MY).

2.6. PCR amplification

The PCR simplex amplification technique using the cytochrome *b* primers targeting mtDNA of 129 bp (Aravindran, 2014) was performed at a final volume of 50 μ l containing 25 μ l of DreamTaq Green PCR Master Mix (2X) (Fermentas, Lithuania), 1 μ l of 5 μ M for each primer (forward and reverse), 21 μ l of nuclease free water (NFW) and 2 μ l of an approximately 100-ng DNA template. Negative and positive DNA controls were prepared by adding 2 μ l of NFW and extracted chicken's DNA from chicken meat, 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 3 min, followed by 45 cycles of heating at 95 °C for 0.5 min, 55 °C for 0.5 min, 72 °C for 0.5 min and a final extension step at 72 °C for 5 min. The amplification

products were electrophoresed through a 3% (w/v) agarose gel in a 1 X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 100 V for 40 min and pre-stained with MaestrosafeTM Nucleic Acid (V-BioScience, MY).

The porcine blood plasm DNA (either fresh or commercial) was amplified using an 161 bp target primer of short interspersed nucleus element (SINE) (Calvo et al., 2001) in a 50- μ l reaction. Volume containing 25 μ l of DreamTaq Green PCR Master Mix (2X) (Fermentas, Lithuania), 1 μ l of 5 μ M for each primer (forward and reverse), 21 μ l of nuclease free water (NFW) and 2 μ l of an approximately 100-ng DNA template. Negative and positive DNA controls were prepared by adding 2 μ l of NFW and Pig Genomic DNA (Novagen[®], Germany), respectively. PCR was also performed in a Mastercycler[®] gradient thermal cycler (Eppendorf, USA) with a temperature program consisting of an initial denaturation at 95 °C for 2 min, followed by 30 cycles of heating at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and a final extension step at 72 °C for 10 min. The amplification products were electrophoresed through a 3% (w/v) agarose gel in a 1 X TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) at 100 V for 40 min and pre-stained with MaestrosafeTM Nucleic Acid (V-BioScience, MY).

The amplification of 1% and 5% (w/w) DNA in commercial blood plasm meatball was performed as described by Laila Liyana et al. (2018). All of the agarose gel electrophoreses of the PCR product used a GeneRulerTM 100-bp DNA ladder (Fermentas, Lithuania) as a molecular size marker and were visualized using a UV Gel Documentation System (Syngene, UK).

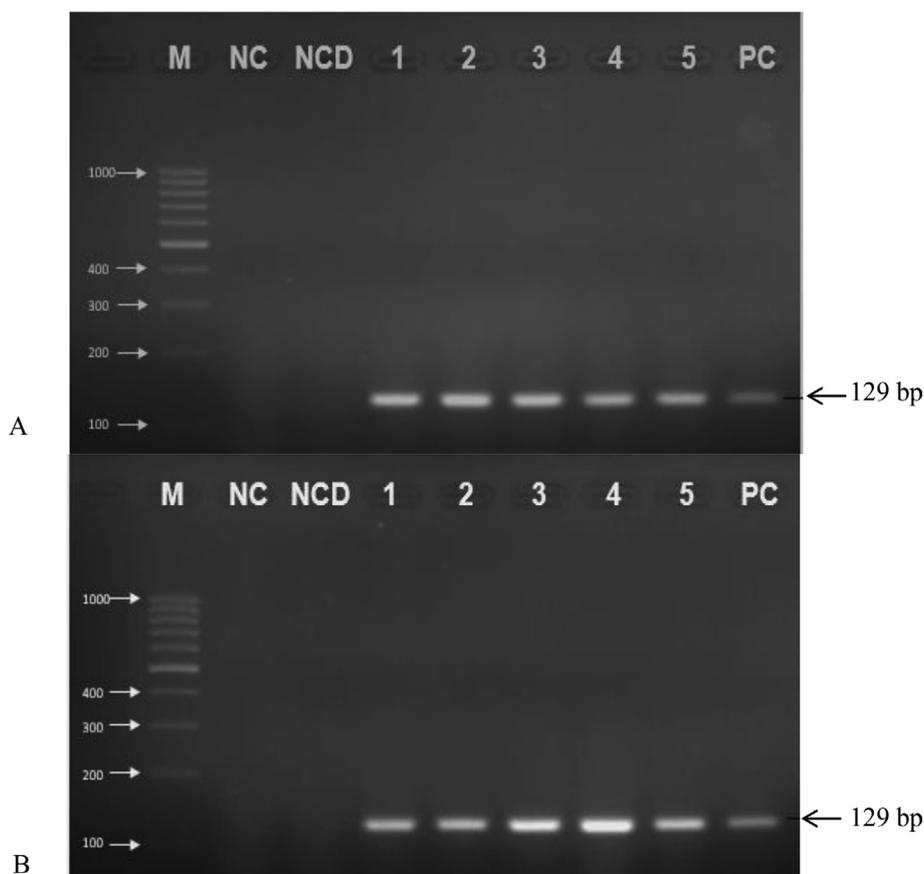


Fig. 1. Amplicons of cytochrome *b* gene fragments (129 bp) for 1% and 5% (w/w) chicken meatball, respectively with different heat treated on 3% (w/v) agarose gel electrophoresis. (A) 1% (w/w) of chicken meat. (B) 5% (w/w) of chicken meat. M: Marker 100 bp DNA ladder, NC: Negative control, NCD: Negative control for DNA extraction, Lane 1: Autoclave, 2: Microwave, 3: Boiling, 4: Roasting, 5: Frying, PC: Positive control (Chicken DNA) (129 bp).

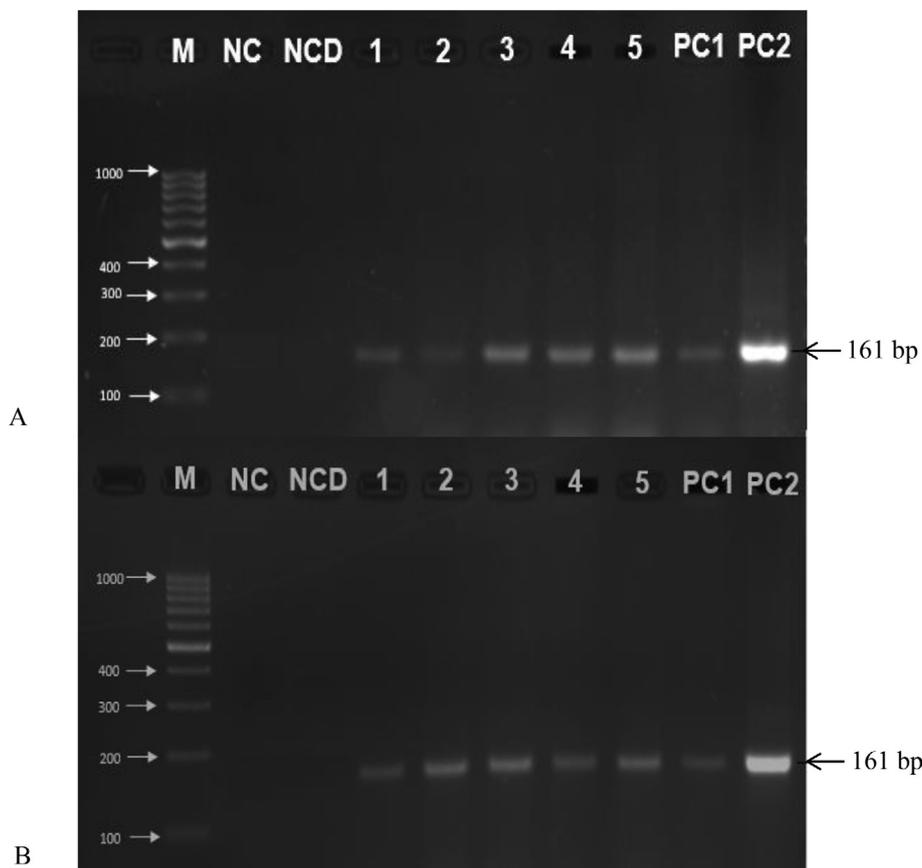


Fig. 2. Amplicons of short interspersed nuclear element (SINE) gene fragments (161 bp) for 1% and 5% (w/w) of chicken meatballs added with 1% and 5% (v/w) fresh porcine blood plasma, respectively on 3% (w/v) agarose gel electrophoresis. (A) Added with 1% (w/w) of fresh porcine blood plasma. (B) Added with 5% (w/w) of fresh porcine blood plasma. M: Marker 100 bp DNA ladder, NC: Negative control, NCD: Negative control for DNA extraction, 1: Autoclave, 2: Microwave, 3: Boiling, 4: Roasting, 5: Frying, PC1: Positive control of (DNA fresh Blood plasma extraction), PC2: Positive control of Pig Genomic DNA (Novagen®, Germany) (161 bp).

3. Results and discussion

Despite blood and its derivatives have been studied as available protein due to its nutritional and functional properties, they are not permissible to be applied in any food products according to *Shariah* Law in Malaysia (MS1500:2019) (Third revision). The purpose of blood plasma added in the processed food product such as meatball is to increase the quality of the product to be consumed which is improved the elasticity, protein and as protease inhibitors (Visessanguan et al., 2000). This study was conducted in attempt to determine the status of chicken meatball (1 and 5% (w/w) chicken meat) when added with 1 and 5% of fresh porcine (v/w) and powder form of commercial (w/w) blood plasma. All chicken meatballs added with fresh porcine (v/w) and powder form of commercial (w/w) blood plasma were heat-treated as indicated in Table 2.

Fig. 1 shows the amplicons of 1 and 5% (w/w) chicken meatball detected using cytochrome *b* (*cyt b*). All heat-treated chicken meatball showed similar intensity and bands of 129 bp in size. The similar intensity may be due to the DNA extraction of the samples were not fully damage the DNA. Thus, DNA is available and serves as target sequence which allows identification of the meat. Our results were consistent as reported by Aravindran et al. (2014) where these primers were successful in targeting mitochondrial DNA (mtDNA) regions of *cytochrome b* in chicken meat.

The results of chicken meatballs added with fresh porcine (v/w) and powder form of commercial (w/w) blood plasma as shown in Figs. 2 and 3, respectively. Similarly as reported by Calvo et al.

(2001), both types of blood plasma produced similar 161 bp of amplicons. The SINE primers were chosen due to its powerful capability to detect porcine DNA in processed and unprocessed food (Calvo et al 2001). Amplification of DNA isolated from 1% and 5% (w/w) chicken meatball added with 1% and 5% commercial porcine blood plasma, respectively using mtDNA such as transfer RNA-ATP8 (tRNA-ATP 8) (Lahiff et al., 2018), did not produce any band (Fig. 4) for 1% (w/v). The possible reason why tRNA-ATP8 as target sequence in blood plasma was nor detected in chicken meat added with 1% (w/v) porcine blood plasma was due to no specific site target sequence obtained in the DNA genome extraction, thus no band was observed for (Fig. 4). However, in 5% (w/v) faded band was observed for chicken ball treated with autoclave, microwave and roasted. This probably due to high percentage of porcine blood plasma added may increase the target sequence allowing the identification of porcine DNA. Whilst, no band was observed for roasting and frying for 5% (w/v) blood plasma may due to the DNA was damaged thus no specific site available for detection. The tRNA-ATP8 primers were useful in detecting porcine DNA from commercial powder gelatin added in heat-processed meatballs (Laila Liyana et al., 2018). Gelatin is a protein-based hydrocolloid which has special and unique characteristics, serving multiple functions with a wide range of applications in various industries including food and beverages, cosmetics and pharmaceutical (Sahilah et al., 2016). The present of mtDNA in gelatin powder is expected due to mitochondria are abundant in the meat cells thus detection of using mitochondria DNA as target sequence in gelatin is possible.

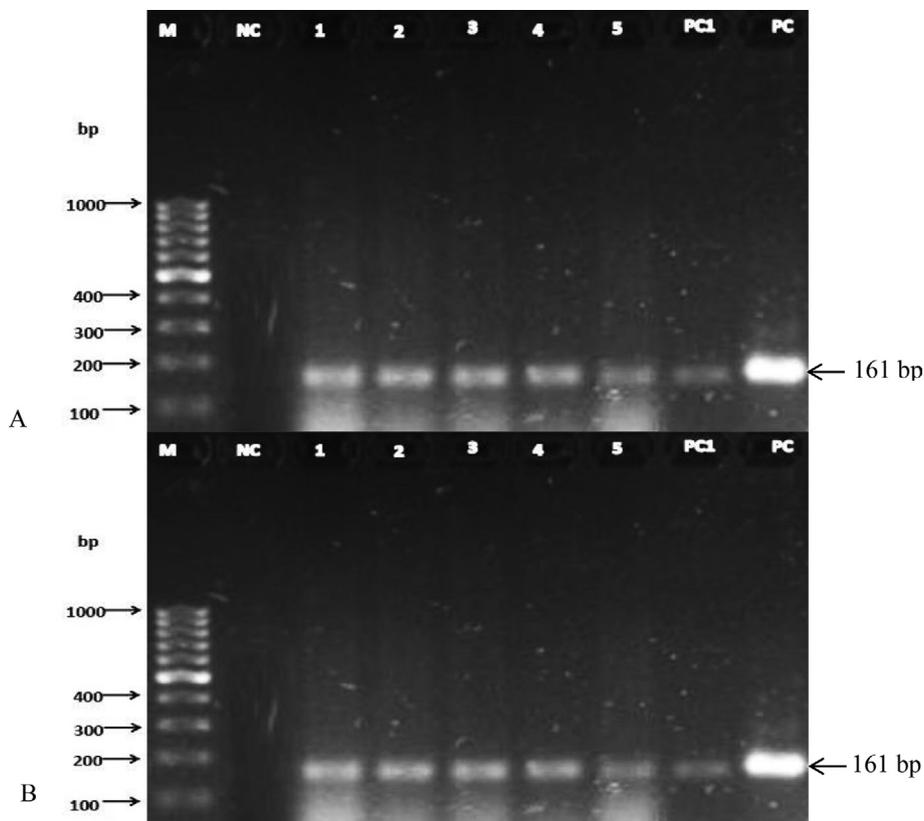


Fig. 3. Amplicons of short interspersed nuclear element (SINE) gene fragments (161 bp) for 1% and 5% (w/w) of chicken meatballs added with 1% and 5% (v/w) commercial porcine blood plasma, respectively on 3% (w/v) agarose gel electrophoresis. (A) Added with 1% (w/w) commercial blood plasma. (B) Added with 5% (w/w) commercial porcine blood plasma. M: Marker 100 bp DNA ladder, NC: Negative control, Lane 1: Autoclave, 2: Microwave, 3: Boiling, 4: Roasting, 5: Frying, PC1: Positive control (powder blood plasma DNA extraction), PC2: Positive control of Pig Genomic DNA (Novagen®, Germany) (161 bp).

The same reason may explain why the mtDNA of tRNA-ATP8 as target sequence in blood plasma is possible to be detected due to the association of mtDNA in blood, though blood originally does not contain nDNA or mtDNA.

In the present study, the nDNA primers of SINE were able to detect both, fresh and commercial porcine DNA from blood plasma (Figs. 2 and 3). The nDNA is originated from the genome of eukaryotic cells. Calvo et al. (2001) examined 55 pig blood samples from nine different breeds and crosses using SINE primers, obtaining 161 bp specific pig band. This result is consistent as described by Calvo et al. (2001), the SINE target sequence gene was detected in the blood plasma which allows identification of animal species. The mtDNA and nDNA are originated from the animal cells not in the blood. The detection of the target sequence from nDNA may come from DNA animal circulating inside the blood.

The intensity of bands in fresh porcine blood plasma (Fig. 2) was not very clear when compared to chicken meat amplicons (Fig. 1). This may probably due to the abundant mtDNA chicken meatball harvested from meat rather than nDNA blood plasma in the form of liquid (Fig. 2). Small quantity of heated-DNA blood plasma may affect the DNA amplification and reduced the amount of amplicons thus, indicated by faded bands (Fig. 2). Comparing between Figs. 2 and 3, unlike the amplicons from commercial porcine blood plasma (Fig. 3) showed thicker bands rather than the fresh blood plasma (Fig. 2). Thicker bands showed more nDNA were available and amplified by the primers therefore, the thick bands were observed.

4. Conclusion

In the present study, the species-specific primers of short interspersed nucleus element (SINE) are easy, sensitive and useful for the detection of DNA porcine in fresh and commercial blood plasma in heat-treated chicken meatball. Thus, the SINE primers could be used to determine the animal species in the processed food as an evidence reveals in this study. This study will assist various parties in food industries to ensure that food choices taken by Muslim community are consistent with the standards set in Islam which prohibits the use of impurities except under compulsory conditions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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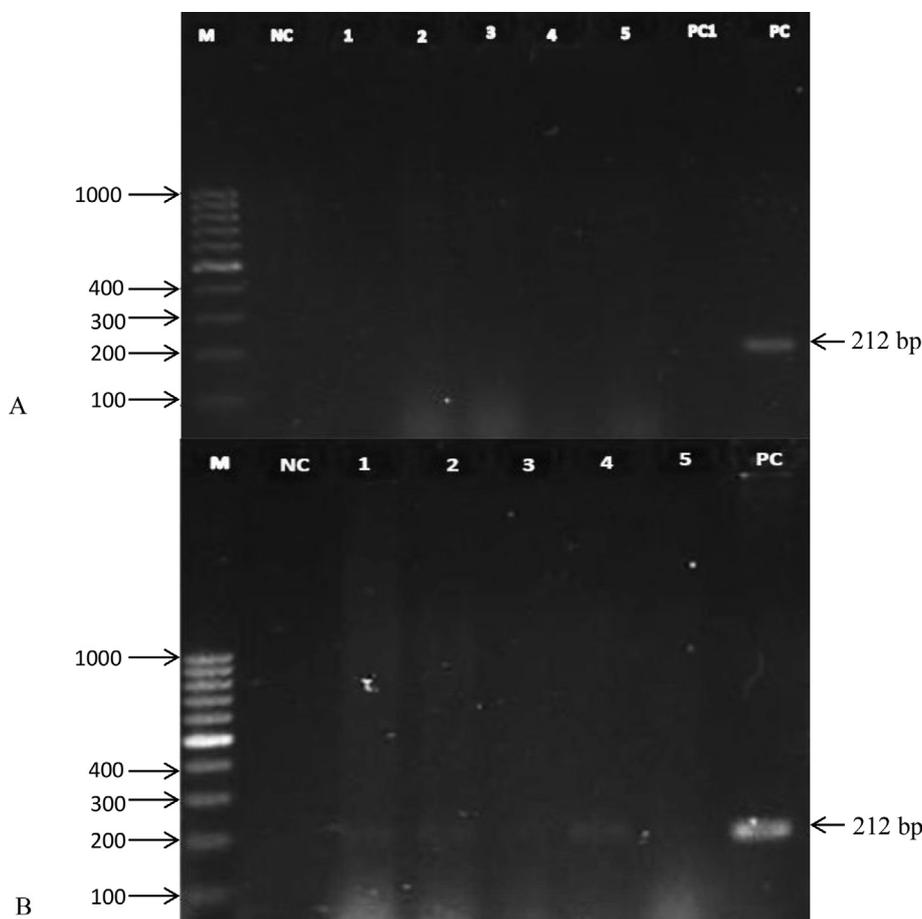


Fig. 4. No amplicons of transfer RNA-ATP8 gene fragments (212 bp) for 1% and 5% (w/w) of chicken meatballs added with 1% and 5% (v/w) commercial porcine blood plasma, respectively on 3% (w/v) agarose gel electrophoresis. (A) Added with 1% (w/w) commercial blood plasma. (B) Added with 5% (w/w) commercial porcine blood plasma. M: Marker 100 bp DNA ladder, NC: Negative control, Lane 1: Autoclave, 2: Microwave, 3: Boiling, 4: Roasting, 5: Frying, PC1: Positive control (powder blood plasma DNA extraction), PC: Positive control of Pig Genomic DNA (Novagen®, Germany) (161 bp).

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