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Meatball model of porcine DNA detection by TaqMan probe real-time PCR

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Abstract

Food adulteration has attracted a great deal of public concern as it affects consumers of different religious beliefs, social backgrounds and economic perspectives. Meat adulteration usually occurs through the substitution of beef with pork due to its lower price and manufacturer's profit motivation. This study was conducted to detect porcine DNA in a spiked meatball model using real-time quantitative polymerase chain reaction (qPCR) targeting the cytochrome B gene through the designing of specific primers and probe sequences. In this study, the amplification efficiency of the designed primers and probe was 95%, with a correlation coefficient (R^2) of 0.997, following amplification with a series of 10-fold serial dilutions of porcine DNA. The specificity of the probe was evidenced by the absence of amplification from DNA of eight different meat species, including chicken, cow, duck, quail, buffalo, rabbit, deer and mutton. Conversely, pork DNA was detected at a threshold cycle of 13.47±0.31, following amplification of 10 ng/µL DNA. In addition, the assay detected a level of pork DNA as low as 0.01 ng/µL in raw spiked beef meatball models and 0.1 ng/µL in heat-treated spiked chicken and beef meatball models. This study is critical, mainly for the Muslim and Jewish communities and individuals who are allergic to pork.

1. Introduction

Food fraud is a global phenomenon that affects consumers worldwide. Food fraud, or adulteration, is defined as the practice of deliberately diminishing the quality of food products, either by substitution, the addition of inferior, and cheaper ingredients or by removing some valuable ingredients (Jha, 2016). Fish and meat products were among the top two product categories with reported cases of food fraud in 2018. In addition, the main type of food fraud related to these products is mislabelling which accounts for 41.89% of instances, followed by adulterations involving the replacement of high-priced meat with lower-priced meat, which accounts for 19.25% (European Commission, 2018). At present, the intentional or unintentional adulteration of meat-based products containing pork DNA has attracted attention among Muslims and Jews due to religious concerns. Furthermore, this practice often leads to unfair trade competition (Raharjo, Chudori and Agustina *et al.,* 2019).

In this regard, the authentication of meat-based food products is critical, due to legislation, public health and religious reasons (Omran *et al*., 2019). Products' labelling which indicates their origin and approved formulation (Fajardo *et al*., 2010) is also taken into account for authentication. Thus, several analytical methods have been developed to identify the species and analyse the authenticity of food products. Commonly used analytical methods, such as DNA-based techniques, are more likely to be used for fraud detection in raw meat species and heat-processed meat products because DNA has better thermal stability during food processing, and has distinct DNA sequences of specific animal species (Rashid *et al*., 2014). Several DNA-based techniques have been used in meat species

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authentication, including randomly amplified polymorphic DNA (Huang *et al*., 2003), restriction fragment length polymorphisms (Doosti *et al*., 2014), species-specific PCR (Kitpipit *et al*., 2013) and real-time PCR (Druml *et al*., 2016). Hamzah *et al*., (2014) reported that conventional PCR can be used to detect porcine DNA in meat products. However, conventional PCR presents some drawbacks, such as it requires end-point analysis. Further, it yields a qualitative outcome, therefore it is less sensitive. In this study, the TaqMan-based real-time PCR assay is used to detect porcine DNA as it is more sensitive, provides a quantitative outcome, while eliminating the need for post-PCR processing (Yusop *et al*., 2012).

Known as *bebola* in Malaysia, meatballs are one of the most preferred meat-based products (Rohman *et al*., 2011) among consumers due to their rich source of animal protein and carbohydrates (Huda *et al*., 2010). Meatballs typically comprise 90% chicken or beef (Aina *et al*., 2019). Some manufacturers seek to maximise their profits by substituting halal beef with cheaper pork when producing meatballs (Montowska and Pospiech, 2011). Previous studies have documented the use of porcine DNA detection in meatball models targeting various mitochondrial genes, such as NADH dehydrogenase subunit 1 (ND-1) (Raharjo *et al*., 2019), and D-loop (Orbayinah *et al*., 2020). The primers and probe sequences designed from the cytochrome B gene sequence have higher interspecies variability compared to *12S rRNA*, *ND2*, *ATPase6* and *16S rRNA* (Mohamad *et al.*, 2013). New species-specific primers and probe sequences were designed in this study, amplifying 121 bp at different regions of the gene, from regions 516 to 637 in the nucleotide of the cytochrome B gene. This model was different from previous studies, in terms of the porcine meat percentage incorporated in the meatball and its exploration of whether the application of a prolonged heat treatment leads to a significant difference in detection capabilities. Furthermore, the meatball model developed in this study was designed to reflect the complex composition of commercial meatballs by enabling the accurate quantification of targets in comparison with binary mixtures of two different species. The specificity and sensitivity of the designed primers and probe sequences on porcine meat DNA were indicated through validation tests with raw meat species and spiked beef and chicken meatball models, respectively.

2. Materials and methods

2.1 Samples

Raw meats (pork, chicken, mutton, duck, quail, buffalo and beef) were purchased from a local butcher shop in Sibu, Sarawak, Malaysia. Rabbit and deer were obtained from Universiti Putra Malaysia (UPM), Bintulu Campus, Sarawak, Malaysia. Spiked meatball models were prepared according to the method detailed by Razzak *et al*. (2015). Raw meats were used for the determination of specificity, and the spiked meatball model was used in the sensitivity of the designed primers and probe.

2.2 In-silico design of primers and probe sequences

The nucleotide sequence of the mitochondrial cytochrome B (*cytb*) gene for pork, chicken, mutton, cow, duck, buffalo, deer, rabbit and quail was obtained from the National Centre of Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov/. The sequences of the mitochondrial *cytb* for various animals were compared and aligned using ClustalW Multiple Alignment tool from Bioedit software (version 7.2.5). The pork-specific primer and probe designated as Pork-F: CAAAGCAACCCTCACACGAT; pork probe: 5HEX -TTACCGCCCTCGCAGCCGTA- 3IABkFQ and Pork-R: AGATTCCGGTAGGGTTGTTG were used in this study. These sequences were designed to amplify 121 bp of the *Sus scrofa domesticus* (pork) mitochondrial *cytb*. The designed primers and probes were verified using the Basic Local Alignment Search Tool (BLAST) in NCBI to ensure the specificity of the designed primers and probe. Mitochondrial *18S rRNA* primers and probe sequences that amplified 140 bp designed by Rojas *et al*. (2011) were included to serve as an internal control for assay validation. The designed primers and probe were synthesised by Integrated DNA Technologies, Singapore.

2.3 DNA extraction and quantitation

DNA from raw meats and meatballs were extracted using DNeasy Mericon Food Kit (Qiagen, German). The extraction process was performed according to a standard protocol for raw meat samples and small fragments protocol for meatball models, with initial samples of 200 mg. The protocols were modified slightly to optimize the quality and quantity of extracted DNA. The purity and concentration of the DNA were determined using Genesys^{TM} 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA). The absorbance of the diluted DNA sample at 260 nm and 280 nm were recorded. DNA integrity was verified by using 1% agarose gel electrophoresis. The gel electrophoresis was performed at 120 V for 30 mins.

2.4 Construction of real-time PCR standard curve

Porcine DNA was diluted into five DNA 10-fold serial dilutions (10^{-1} until 10^{-5}) for the construction of the standard curve. qPCR assay was carried out based on the

protocol for GoTaq Probe qPCR Master Mix product information (Promega, USA), with a slight modification in terms of total volume. Master Mix containing 5 µL of 5× GoTaq Probe qPCR Master Mix (Promega, USA), 0.25 µL of 20 µM of each forward and reverse primer, 0.125 µL of 20 µM of hydrolysis TaqMan probe and 3.375 µL nuclease-free water were prepared. Then, 1 µL of DNA template was added to each respective PCR tube which made up a final volume of 10 µL. Five tubes containing DNA templates with 10-fold serial dilutions were run simultaneously in triplicates. The reaction mixtures without template or NTC were included as the negative control. The PCR tubes were centrifuged briefly to ensure the reagent components remain at the bottom of the tube prior to loading into DTprime Real-time thermal cycler (DNA-Technology, Russia). Real-time PCR was operated according to the recommended thermal cycling condition as such, GoTaq activation at 95°C, 2 mins for 1 cycle; denaturation at 95°C, 15 s; annealing/extension at 60°C, 1 min, both for 40 cycles. The linearity, efficiency and sensitivity of the qPCR were determined by plotting the standard curve. The standard curve was plotted as threshold cycle (C_t) values against the logarithm of the template concentration.

2.5 Determination of specificity of the designed primers and probe sequences

The specificity of the designed primers and probe for TaqMan qPCR assays were determined using the DNA template of the raw meat samples. The reaction and thermal cycling conditions were carried out according to the method described in Section 2.4. A no-template control (NTC) containing nuclease-free water was added to the NTC tube which acted as a negative control. Internal control or positive control was prepared by adding *18S rRNA* primer and probe to the master mix. qPCR reaction was done in triplicates for two independent studies.

2.6 Preparation of spiked meatball model

The chicken and beef meatball model was prepared according to Razzak *et al*. (2015), with a slight modification in the amount of spiked pork meat. Table 1 indicates the formulation for chicken and beef meatballs. The prepared chicken and beef meatballs were spiked with 10% (w/w) of pork meat individually and collectively to make ≥35g of meatballs per piece. The amount of minced chicken and beef used to make each meatball was similar. Another set of prepared meatballs was boiled at 100°C for 2 hrs and 30 mins. The DNA extraction and quantitation were done based on the method described in Section 2.3.

Table 1. Formulation for spiked chicken and beef meatballs

Ingredients (g)	Meatball (≥35g/ piece)	
	Chicken	Beef
Minced meat	23	23
Soy protein	3	3
Starch/ bread crumb	5	5
Chopped onion[a]	1	1
Chopped ginger[a]	0.1	0.1
Cumin powder[a]	0.75	0.75
Garlic powder[a]	0.5	0.5
Black pepper[a]	0.14	0.14
Tomato paste	1.5	1.5
Butter	1.5	1.5
Salt	SA[b]	SA[b]
Flavour Enhancer	SA[b]	SA[b]

[a]The amounts were in approximate value and some ingredients were taken in teaspoon measurements.
[b]SA = suitable amounts.

2.7 Determination of detection limit of designed primers and probe sequences

The detection limit was determined using DNA extracted from raw and heat-treated beef and chicken spiked meatball models. DNA was extracted from beef and chicken spiked meatballs containing 10% (w/w) of pork meat. Then, 10 ng/µL DNA extracted from each beef and chicken spiked meatball was serially diluted 10-fold from 10 ng/µL to 0.0001 ng/µL. The step was repeated with a heat-treated spiked meatball to determine the effect of heat treatment on this assay. qPCR reaction was done in triplicates for two independent studies. The reaction and thermal cycling conditions were carried out according to the method described in Section 2.4. The detection rate of the designed primers and probe were determined using Equation 1 (Cai *et al.,* 2017). The detection rate was calculated for each meatball spiked model and pork meatball model.

$$\text{(Positive detection) PP, BP, CP / (N)} = \text{DR} \quad (1)$$

Where the number of tubes having positive detection for porcine DNA in sample replicates was calculated; PP is positive detection for porcine DNA in pork sample replicates; BP is positive detection for porcine DNA in beef sample replicates; CP is positive detection for porcine DNA in chicken sample replicates; N is the total number of tubes of sample replicates; DR is the detection rate.

2.8 Statistical analysis

Statistical analysis was conducted using Student's t-test (IBM SPSS Statistics Version 20) to determine the differences between the C_t values of the raw spiked meatball models and heat-treated spiked meatball models.

3. Results and discussion

3.1 Multiple sequence alignment of species used in the study and primer probe design

The nucleotide sequence of the mitochondrial cytochrome B (*cytb*) gene for *Sus scrofa domesticus* (NC_012095.1), *Gallus gallus* (NC_040970.1), *Ovis aries* (NC_001941.1), *Bos taurus* (NC_006853.1), *Anas platyrhynchos* (NC_009684.1), *Bubalus carabanensis* (NC_006295.1), *Rusa unicolor* (NC_031835.1), *Oryctolagus cuniculus* (NC_001913.1) and *Coturnix japonica* (NC_003408.1) were obtained from the NCBI database. Figure 1 shows multiple alignments of the sequences of the mitochondrial *cytb* for various animals using ClustalW Multiple Alignment tool (Bioedit software version 7.2.5). The non-conserved region of *cytb* sequences of *Sus scrofa domesticus* among the others was selected as primers and probes to ensure the primers and probes were specific for pork. In this study, Taqman probe qPCR assay was designed to amplify a short amplicon length of 121 bp. This is in agreement with Lim *et al*. (2011) who reported that amplification of less than 150 bp increases qPCR efficiency. Thus, a shorter amplicon provides higher chances of detection, reduces the chances of secondary structure formation (Toouli *et al*., 2000), and ensures the applicability of sequence detection in processed food products, particularly those that have been subjected to thermal treatment. The incorporation of Taqman probe with species-specific primer sequences in food matrices improves the consistency of the assay (Köppel *et al*., 2011).

Figure 1. Multiple sequence alignment of the mitochondrial *cytb* for various animals in this study

3.2 Quantification of DNA quantity and quality

The A_{260}/A_{280} ratio provides an insight into the purity of the extracted DNA. In this study, the results were reported based on the data from two independent studies. The possible contaminants of DNA comprise protein, polyphenols, polysaccharides and other PCR inhibitors (Piskata *et al*., 2019). Four samples namely lamb, duck, rabbit and deer were reported with A_{260}/A_{280} absorbance ratio that is lower than 1.7. Meanwhile, no DNA sample was reported for an absorbance ratio of more than 2.0. A_{260}/A_{280} higher than 2.0 usually indicates RNA contamination (Piskata *et al*., 2019) whereas a ratio below 1.7 indicates protein contamination. The residual impurities carried over from the DNA extraction process such as ethanol or phenol can also lower the A_{260}/A_{280} of the extracted DNA. Laube *et al*. (2007) reported that the DNA yield of the extracted DNA can be affected by the sources of tissues. The DNA extracted from fatty tissue contains a lower concentration of DNA compared to DNA extracted from the kidney, liver, heart and tendon tissues. In addition, butter used in producing the meatball model may lower the DNA yield as high lipid content affects DNA extraction (Costa *et al*., 2010). Thus, optimisation has been done in this study to obtain a high yield of DNA concentration sufficient for qPCR assay.

In this study, the quality of the extracted DNA can be verified by electrophoresis analysis through a 1% agarose gel. All the DNA extracted from the raw meat was of good quality as the DNA band remained intact. For mutton, duck, rabbit, buffalo and quail sample, the DNA band appeared thicker as the concentration of the DNA was higher (data not shown). However, all the DNA extracted from heat-treated meatball models and raw spiked chicken meatball models were degraded as it appeared as an expanded smear with some of the fragmented DNA band. This is in agreement with Piskata *et al*. (2017) who stated that heat, physical or chemical treatment may negatively affect the quality and quantity of the extracted DNA as it will result in smearing and fragmentation of the extracted DNA. Furthermore, Malentacchi *et al*. (2014) reported that the sample quality, sample age, repeated freezing-thawing, retention to the tubes and storage condition can significantly affect the DNA integrity. Instead, newly short amplicon primer and probe sequences designed in this study are workable to detect porcine DNA in the spiked meatball model. This could be due to the availability of certain DNA fragments which may contain the target sequences, ensuring successful detection by the short amplicon. Hence, these sequences provide an alternative by which the chances of detection could be increased through amplification of the DNA fragment from heat-treated food products.

3.3 Construction of standard curve for primers and probe

The prerequisite step for conducting qPCR assays is to construct a standard curve for the target gene. The performance of the subsequent qPCR assays can be

estimated through a standard curve and it is used to determine the efficiency of the designed primers and probe. The optimal correlation coefficient (R^2) of a standard curve should be more than 0.99 as it measures how well the data fit on the standard curve. The values of the slope between -3.1 and -3.58 are acceptable, which correspond to the qPCR amplification efficiency ranging between 90 to 110% (Bio-Rad Laboratories, 2006; Science Squared, 2016). Based on Figure 2, C_t was plotted against $\log_{10}$ of five different 10-folds DNA dilutions (10, 1, 0.1, 0.01 and 0.001 ng/µL) without detection for the non-template control (NTC), which indicates the absence of contamination and primer-dimer. The amplification of five DNA 10-fold serial dilutions showed a good linear regression and correlation coefficient (R^2) of 0.997. The assay indicates an acceptable value of 95% efficiency which corresponds to -3.431 slope value. Thus, the reliability of the designed *cytb* primers and probe in detecting porcine DNA at different concentrations was established. In addition, Figure 3 shows the C_t plotted against $\log_{10}$ of five different 10-folds DNA dilutions (10, 1, 0.1, 0.01 and 0.001 ng/µL), without amplification observed for NTC. The amplification of the five DNA 10-fold serial dilutions showed a very good linear regression and correlation coefficient (R^2) of 0.9909. The assay indicates an acceptable value of 97% efficiency which corresponds to -3.381 slope value. Hence, the result indicates that the internal control used in this study has high efficiency and reproducibility.

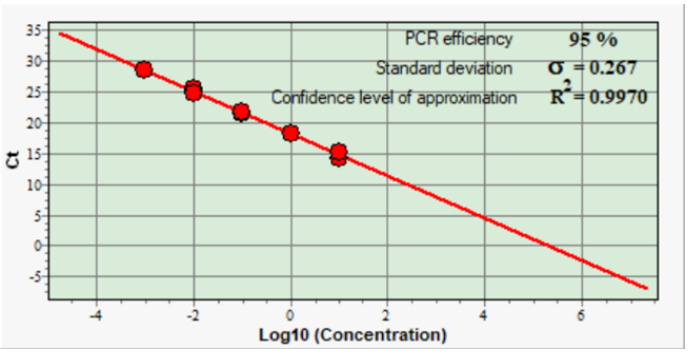

Figure 2. Correlation between logarithms of porcine DNA concentration (x-axis) with C_t value (y-axis) for *cytb*

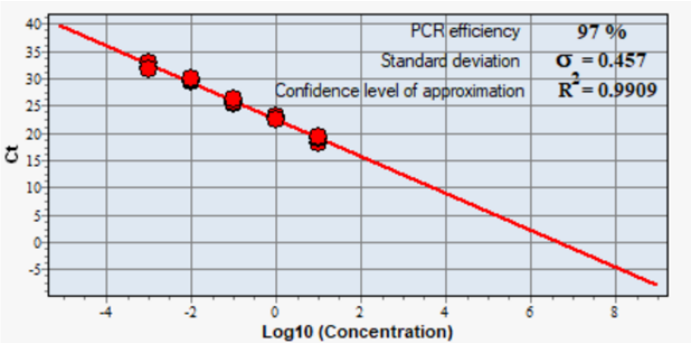

Figure 3. Correlation between logarithms of porcine DNA concentration (x-axis) with C_t value (y-axis) for *18S rRNA*

3.4 Determination of the specificity of the primers and probe

Specificity of the designed *cytb* primers and probe against porcine DNA was validated with eight meat species namely chicken, beef, duck, mutton, buffalo, deer, rabbit and quail through qPCR assay. The qPCR assays for the validation of specificity were conducted in triplicates for two independent studies. The designed *cytb* primers and probe is porcine-specific as indicated by the absence of amplification against DNA extracted from the other eight species. The specificity of the mitochondrial *18S rRNA* primers and probe designed by Rojas *et al*. (2011) was determined to validate the presence of eukaryotic DNA that cannot be detected and amplified by porcine specific PCR system (Kim *et al.,* 2016). The *18S rRNA* is a structural ribosome for the small element of eukaryotic ribosomes, responsible for synthesizing protein and serves as the basic components of all eukaryotic (Uddin and Cheng, 2015). These universal sequences designed within *18S rRNA* positively detect eukaryotic cells and preclude false negative outcomes. It also helps to confirm the functionality of the reaction mix, factors that might affect the real-time PCR amplification process and the possibility of false-positive detection in the qPCR (Hossain *et al*., 2017). Table 2 shows the mean C_t value of the amplified DNA for specificity assay using the designed *cytb* primers and probe and *18S rRNA*. Figures 4 and 5 show the representative qPCR amplification curve for the *cytb* and *18S rRNA* specificity assay, respectively. DNA amplification was observed for all species which indicates the *18S rRNA* primers and probe as highly specific to eukaryotic DNA.

3.5 Determination of sensitivity for the newly designed cytb primers and probe

The sensitivity of primers and probes in qPCR assay is vital as it provides an insight into the suitability and capacity of a detection system. In this study, the limit of detection (LOD) was determined to establish the lowest concentration of porcine DNA that can be detected by the designed primers and probe. LOD is defined as the lowest concentration that provides a positive result in all sample replicates corresponding to at least a 95% detection rate (Cai *et al*., 2017). A spiked meatball model was prepared to represent the complex matrices of the commercially processed meat products. The sensitivity of the assay was determined using 10-fold serially diluted raw and heat-treated beef and chicken spiked with pork meatball. The experiment was repeated for two independent studies, each in triplicates. Raw meatballs and heat-treated pork meatballs served as a control for comparison purposes. The LOD of the qPCR assay for

Table 2. Mean C_t value of two independent studies for *cytb and 18S rRNA* specificity assay

Species DNA[a]	Mean C_t [b] ± SD[c]	Number of Positive Replicates	Mean C_t [b] ± SD[c]	Number of Positive Replicates
	cytb		*18S rRNA*	
Pork	13.47 ± 0.31	6/6	17.17±1.99	6/6
Chicken	40±0.00[d]	0/6	17.6±1.19	6/6
Beef	40±0.00	0/6	17.1±0.77	6/6
Duck	40±0.00	0/6	16.73±1.12	6/6
Mutton	40±0.00	0/6	17.97±0.72	6/6
Buffalo	40±0.00	0/6	17.2±0.55	6/6
Deer	40±0.00	0/6	16.78±0.70	6/6
Rabbit	40±0.00	0/6	17.53±0.80	6/6
Quail	40±0.00	0/6	15.6±1.14	6/6

[a]Similar DNA concentration (10 ng/µL) was used for various meat species.
[b]The C_t values were the mean of replicate assays (n = 6).
[c]SD: Standard deviation
[d]40±0.00 : Porcine DNA not detected after cycle 40

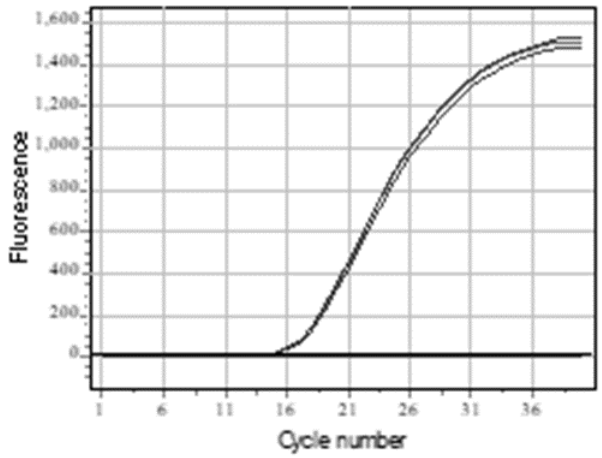

Figure 4. qPCR amplification curve of the *cytb* specificity assay

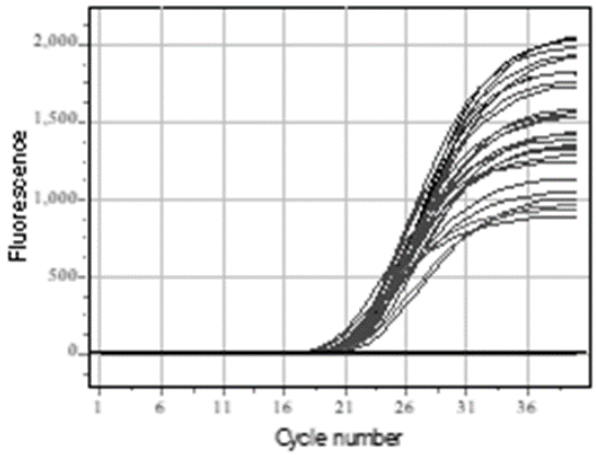

Figure 5. qPCR amplification curve for *18S rRNA* specificity assay

DNA extracted from raw and heat-treated meatball models is stated in Table 3. In this study, the LOD for raw spiked beef and chicken meatballs were 0.01 ng/µL and 0.1 ng/µL, respectively. In comparison, the LOD of raw pork meatball is 0.001 ng/µL as it consists of solely pork meat and serves as a control.

LOD of the heat-treated meatball model was determined using the meatball model subjected to boiling at 100°C for 2 hrs and 30 mins. The LOD of heat-treated spiked chicken and the beef meatball was 0.1 ng/µL. Furthermore, the LOD of the heat-treated spiked meatball model is lower than the LOD of the raw meatball model. According to Bhat *et al*. (2016) although DNA has a stable structure compared to protein, DNA present in the food matrices may be damaged and disintegrated into smaller pieces when subjected to heat treatment. Processing and intensive cooking may lower the concentration of the DNA as, during meat processing or heating, the cellular membrane of the meat is disrupted. A previous study by Ali *et al*. (2012) reported a detection limit of 0.01% (w/w) of pork in a beef meatball spiked model following boiling in water for 15 mins. In comparison with the present study, prolonged boiling of adulterated meatballs in water does not significantly affect the detection limit.

Table 3. Limit of detection for raw and heat-treated adulteration meatball model

Concentration (ng/µL)	Raw spiked meatball									Heat-treated spiked meatball								
	BP	BN	DR	CP	CN	DR	PP	PN	DR	BP	BN	DR	CP	CN	DR	PP	PN	DR
10	6	0	100	6	0	100	6	0	100	6	0	100	6	0	100	6	0	100
1	6	0	100	6	0	100	6	0	100	6	0	100	6	0	100	6	0	100
0.1	6	0	100	6	0	100	6	0	100	6	0	100	6	0	100	6	0	100
0.01	6	0	100	3	3	50	6	0	100	3	3	50	2	4	33.3	5	1	83.3
0.001	0	6	0	0	6	0	6	0	100	0	6	0	0	6	0	1	5	16.7
0.0001	0	6	0	0	6	0	1	5	16.7	0	6	0	0	6	0	0	6	0

BP: Beef Positive, BN: Beef Negative, CP: Chicken positive, CN: Chicken negative, PP: Pork Positive, PN: Pork Negative, DR: Detection Rate (%)

3.6 Statistical analysis

The statistical analysis between raw and heat-treated spiked meatball model was determined using Student's t-test. As shown in Table 4, no significant difference ($p>0.05$) was observed in the porcine DNA detection between raw and heat-treated spiked chicken and beef meatballs. This result is in agreement with López-Andreo *et al*. (2012) who concluded that heat treatment does not significantly affect the result of qPCR against the different concentrations of DNA. However, there was a significant difference in mean C_t values between raw and heat-treated pork meatballs at 10 to 0.01 ng/µL as the p values are less than 0.05. According to Hird *et al*. (2006), although heat-treatment may reduce the amount of detectable DNA, the DNA extracted from a heat-treated sample can still be detected as the primers used in qPCR targeting only a short DNA fragment.

4. Conclusion

Consumption of food products that are adulterated with pork or its derivatives is forbidden for Muslims and Jews. In addition, adulterated foods can also cause adverse health effects to the individual who is prone to an allergic reaction against certain meat spp. Therefore, knowledge of food science and technology is critical to counter food adulteration issues. In this study, the presence of porcine DNA was detected through qPCR assay using the designed *cytb* primers and probe sequences. Having an amplification efficiency of 95%, the designed primers and probe are specific to porcine DNA as no other species were detected by the assay. Moreover, it can detect as low as 0.01 ng/µL and 0.1 ng/µL of pork DNA in raw spiked beef and chicken meatball model, respectively. Pork DNA was detected at a concentration of 0.1 ng/µL in the heat-treated spiked chicken and beef meatball model. This study shows that the extracted DNA following prolonged meatballs heat treatment does not affect qPCR, which was comparable with other meatball models that were only subjected to boiling for 15 mins. Other thermal treatment processing parameters such as using a microwave oven could be incorporated in the future study to observe the effect of different conditions on detection limits. Furthermore, the designed primers and probe can be further applied for detection of porcine DNA in commercial beef and poultry canned food products with bogus *Halal* logo and without *halal* logo. Finally, as the designed primers and probe are sensitive and specific in detecting the presence of porcine DNA in raw and heat-treated products, it has the potential to be used in the screening for food adulteration and mislabelling which will protect consumers from food fraud.

Conflict of interest

The authors declare no conflict of interest.

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Table 4. Statistical analysis of raw and heat-treated spiked meatball model

C_t^*±SD# for meatball model		Concentration (ng/µL)					
		10	1	0.1	0.01	0.001	0.0001
C_t^*±SD# for raw meatball	Beef	17.8±0.84[a]	21.6±0.83[a]	27.1±0.91[a]	31.0±1.00[d]	40±0.00	40±0.00
	Chicken	17.1±1.18[a]	21.1±1.26[a]	27.0±1.60[a]	35.2±5.55[a]	40±0.00	40±0.00
	Pork	14.6±0.91[b]	18.2±0.86[b]	22.2±0.8[b]	26.8±1.81[b]	31.6±1.31[b]	38.8±2.90[a]
C_t^*±SD# for heat treated meatball	Beef	18.3 ±0.65[a]	22.8±1.55[a]	27.2±1.19[a]	36.3±4.20[e]	40±0.00	40±0.00
	Chicken	17.9± 0.45[a]	22.5±1.33[a]	28.0±2.09[a]	37.8±3.44[a]	40±0.00	40±0.00
	Pork	16.1±0.30[c]	20.0±0.90[c]	24.8±0.59[c]	31.1±4.43[c]	38.9±2.82[c]	40±0.00[a]

Values were expressed as average mean±standard deviation. Values with the same superscript within the same column were not significantly different at $p>0.05$

*The C_t values were the mean of replicates assays that indicated positive results.

#SD: Standard deviation.

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