FOOD
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Detection of pork in processed meat products by species-specific PCR for halal verification: food fraud cases in Hat Yai, Thailand

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Abstract

Consumer confidence in halal integrity of the unique and various food products provides Hat Yai, Thailand a great potential for a global destination of Muslim-friendly tourism. Islam prohibits the consumption of pork and its derivatives in any food products. The issue of food adulteration and contamination, particularly in the processed halal meat products with pork and its derivatives, greatly concern Muslim consumers. The aim of this study was to detect the presence of pork DNA from processed meat products collected from self-proclaimed "halal" Muslim street food stalls at Hat Yai, Thailand. Thirty-six samples of various processed meat products were randomly collected from seven Muslim street food stalls including patties, meatballs, and sausages containing processed chicken, beef, or a mixture of various meats. The detection of the presence of pork and its derivatives was performed by a conventional polymerase chain reaction (PCR) technique based on the pork-specific primers for a conserved region in the mitochondrial (mt) 12S ribosomal RNA (rRNA) gene. The results revealed that three out of the thirty-six samples (8.3%) were positively identified to contain porcine DNA by the detection of the expected single band of size 387 bp. The DNA method conveniently provides reliable results for routine food analysis for halal requirement. Overall, the study highlights the importance of halal integrity between the producers, suppliers, and street food business owners to provide halal food products particularly to Muslim consumers.

1. Introduction

The uniqueness of various local foods has made Hat Yai, Thailand to be known as one of the popular tourism destinations particularly among the Muslim tourists (Kuncharin and Mohamed, 2013). As it is potentially be expanded to become a global Muslim-friendly tourism destination, the great concern is therefore about seeking for halal food. As Muslim is a minority population in Thailand, consumer confidence on food products that are "self-proclaimed" as halal from Muslim street food stalls is solely based on trust. Nevertheless, halal integrity of food products should not be compromised by any non-compliance of halal requirements particularly concerning the source or origin of the products. Muslim seller is therefore highly responsible to ensure the food products are halal from sources of their origin.

Many studies indicated that the significant influence factor in the actual purchasing intention of halal products is predominantly driven by the consumer confidence towards the halal brands (Shaari et al., 2010; Mohayidin

and Kamarulzaman, 2014). The increased awareness on the issue of food authenticity is significantly influenced by the consumers' knowledge on the food products (Ruslan et al., 2018). The principle of shariah (law) in Islamic practice strictly prohibits (haram) the consumption of pork and its derivatives in any materials to be used particularly in foods (Fadzlillah et al., 2011). The complexity of modern supply chain and globalisation of food production is one of the many possible routes of halal food contamination with non-halal substances. In particular, the many recent reports on food adulteration happen globally with ambiguous or multispecies contaminants from non-halal sources have generated great concern among the Muslim consumers (Doosti et al., 2014; Di Pinto et al., 2015).

The detection and identification of the presence of pork and its derivatives in food products can be performed by two methods namely protein and DNA analysis (Rahmati et al., 2016). Protein-based analytical methods include immunological assay, chromatography

and peptide examination (Ha *et al*., 2017). Nevertheless, in protein analysis, the method is limited by the nature of protein itself as it is highly sensitive and easily denatured by high temperature during processing (Rahmati *et al*., 2016). In turn, DNA-based techniques offer more robust, simple and rapid detection of pork and its derivative compared to protein-based analysis (Ha *et al*., 2017). The DNA-based methods include conventional polymerase chain reaction (PCR), real-time PCR, PCR-restricted fragment length polymorphism (PCR-RFLP), and species-specific PCR (Calvo *et al*., 2002; Aida *et al*., 2005; Karabasanavar *et al*., 2014; Chai *et al*., 2017). The method could effectively work for processed meat samples to give reliable identification and conclusion. PCR identification of species using mt-DNA provides a number of advantages. With a very small amount of sample, mt-DNA provides higher detection sensitivity and specificity as it contains a high number of copies per cell (Montiel-Sosa *et al*., 2000; Che Man *et al.*, 2007). The aim of this study was to detect the presence of pork DNA using a conventional PCR in processed meat products available in self-proclaimed "halal" Muslim street food stalls at Hat Yai, Thailand.

2. Materials and methods

2.1 Processed meat samples

A total of 36 processed meat samples were obtained from 7 Muslim street food stalls that were located at a far distance from each other in the area of Hat Yai, Thailand. The selection of the stalls was based on the "halal" labelled displayed in front of the stalls. The samples include patties, meatballs, and sausages containing processed chicken, beef, or a mixture of various meats. Each sample was kept in a separate plastic container to avoid cross-contamination between the samples. Samples were stored at -20°C before the DNA extraction procedure to prevent the enzymatic degradation of DNA.

2.2 DNA extraction

The extraction of genomic DNA from the processed meat samples was performed according to the manufacturer's protocol using the Genomic DNA Mini Kit (Tissue) (Geneaid, Taiwan). About 30 mg of the sample was cut and then transferred to a 1.5 mL microcentrifuge tube. 200 µL of GT Buffer was added to the tube and vortexed vigorously. 20 µL of Proteinase K was added to the sample and then vortexed vigorously. The mixture was incubated at 60°C for 30 mins and the tube was inverted and vortexed for every 5 mins during the incubation. 200 µL of GBT Buffer was added and vortexed vigorously. The tube was incubated at 60°C for 20 mins. The tube was inverted and vortexed for every 5

mins during the incubation. To remove the insoluble material, the mixture was centrifuged for 2 mins at 14,000 x *g* then the supernatant was transferred to a new 1.5 mL microcentrifuge tube. 200 µL of absolute ethanol was added to the lysate and then immediately vortexed. The sample was transferred to the GS Column then centrifuged at 14,000 x *g* for 2 mins. The Collection Tube (previously attached with GS Column) was discarded and GS Column was transferred to a new collection tube. 400 µL of W1 Buffer was added to the GS Column then centrifuged at 14,000 x *g* for 30 s. The flow-through was discarded and the GS Column was put back in the Collection Tube. Wash Buffer of 600 µL was added to the GS Column and centrifuged at 14,000 x *g* for 30 s. The flow-through was discarded. To dry the column matrix, the GS Column was centrifuged further at 14,000 x *g* for 3 mins. The dried column was transferred to a clean 1.5 mL microcentrifuge tube. Preheated Elution Buffer of 50 µL was added to the center of the column matrix and let to stand for 5 minutes to ensure the Elution Buffer was completely absorbed. Finally, the column was centrifuged at 14,000 x g for 30 s to elute the purified DNA and kept at -20°C for further analysis.

2.3 Oligonucleotide primers and Polymerase Chain Reaction (PCR) amplification

Primers of two sets used were from the previous studies published by Rodríguez *et al.* (2003), and Ha *et al.* (2017) (Table 1). Amplification of the mt cyt *b* gene was performed in a final volume of 25 µL containing the extracted DNA, PCR SuperMix (Invitrogen), each forward and reverse primers. Raw meat samples of pork, chicken and beef served as a reference were firstly tested for both sets of the primers to evaluate the specificity of the primers (Table 1). For the processed meat samples, only one set of primers were utilized for the genomic DNA amplification. Amplification of the genomic DNA was performed with a T100 Thermal Cycler (Bio-Rad) according to the optimized programme under the following conditions: an initial denaturation step of 94°C for 2 mins to completely denature the DNA template, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 68°C for 30 s and extension at 72°C for 1 min. The final cycle for complete synthesis of elongation of DNA molecules was followed by final extension at 72°C for 5 mins. A total of 10 µL of PCR products was electrophoresed at constant voltage (100 V) on 1 % agarose gel (Invitrogen) for 30 mins in 1x TAE buffer and stained by SYBR Safe DNA gel stain (Invitrogen). A 3000 bp DNA ladder was used as size reference. The gel photo was taken using molecular imager Gel Doc XR+ (Bio-Rad).

Table 1. Primer sequences specific for the pig mitochondrial DNA D-loop

Primers	Sequence primers (5' → 3')	Length of base pair (bp)	Length of PCR product (bp)	References
12SFW	CCA CCT AGA GGA GCC TGT TCT ATA AT	26	387	Rodríguez *et al.* (2003)
12SR	GTT ACG ACT TGT CTC TTC GTG CA	23		
Pork-F	GGT TCT TAC TTC AGG ACC ATC	21	294	Ha *et al.* (2017)
Pork-R	GTG TAC GCA CGT GTA TGT AC	20		

2.4 Validation

All the positive samples priory identified to contain pork DNA were re-processed and analyzed to validate the initial screening results. The samples were taken (i.e. from the different portion or site of the previously taken samples) for genomic DNA extraction as previously described. The DNA products were amplified by PCR and analyzed by agarose gel electrophoresis. The results were compared with the previous screening results for validation.

3. Results and discussion

Halal authentication is greatly essential to ensure that the integrity of the halal status of food products is preserved. Genomic method using PCR amplification of species-specific DNA for the detection of food adulteration has received great attention due to its significant advantages. In contrast to other detection methods, the DNA-based method is robust, sensitive and specific for various types of food products including processed meats (Rahmati *et al.*, 2016). Compared to protein, DNA is relatively stable in high-temperature conditions thus makes it a valuable and convenient material for detection (Rahmati *et al.*, 2016). Therefore, many current studies have been specifically focusing on DNA-based method as it provides a more effective approach to detect and identify pork adulteration.

The extracted genomic DNA from the raw meat and processed meat samples produced a good quality of DNA products in term of its concentrations (ng/μL) and purities (260/280) as indicated by the nanodrop analysis (data not shown). The results showed that all the DNA products were acceptable and sufficient to be used as the template for PCR amplification for the detection of pork DNA. Initially, two sets of specific primers for pork mitochondrial DNA were tested against raw meats of reference samples of pork, chicken, and beef (Rodríguez *et al.*, 2003; Ha *et al.*, 2017). PCR amplification products of 12SRNA gene of primers by Rodríguez *et al.* (2003) yielded a single band of size 387 bp while from the Ha *et al.* (2017) produced a single band of size 294 bp in raw pork sample (Figure 1). There were no bands detected from the raw samples of chicken and beef indicated that the primers were specific to pork species (Figure 1). In further analysis, primers by Rodríguez *et al.* (2003) were

selected to be used to detect pork DNA adulteration in all the processed meat samples.
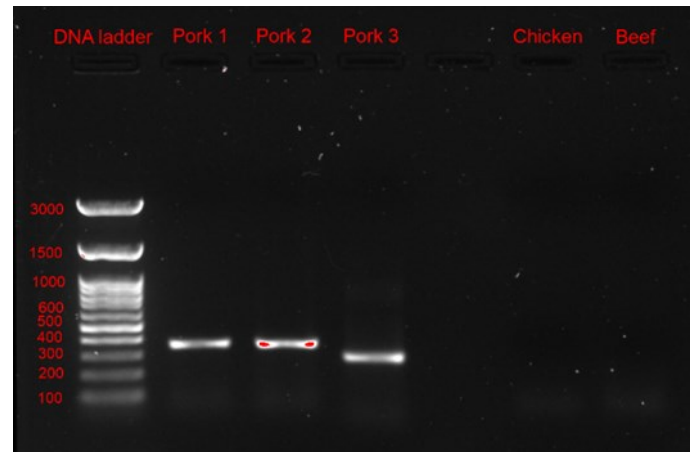

Figure 1. Agarose gel electrophoresis of PCR products from pork, chicken and beef. Samples of Pork 1 and Pork 2 were amplified using primers by Rodríguez *et al.* (2003) yielded a single band of size 387 bp. Sample of Pork 3 was amplified from primers by Ha *et al.* (2017) produced a single band of size 294 bp. Chicken and beef were amplified using primers by Rodríguez *et al.* (2003) produced no band.

In the initial screening of the presence of pork, the agarose gel electrophoresis analysis of PCR products revealed that 8 of the processed meat samples were identified to produce a specific single band of pork of the expected size 387 bp (Table 2). Samples of D-18 and F-29 showed thick and clear specific single bands of pork (Figure 2). For the other 6 samples, B-6, B-7, B-8, B-10, F-28, and F-30 the identified specific single bands observed were faint and not so clear (Table 2). From the results, we assumed that the 8 processed meat samples were positive to contain pork DNA but required further analysis for validation. On the other hand, there were no bands detected from the rest of 28 processed meat samples which indicated the absence of pork or its derivatives.

To validate the results, all the processed meat samples (B-6, B-7, B-8, B-10, D-18, F-28, F-29 and F-30) that were identified positive to contain pork adulteration in the initial screening stage were again extracted for its DNA, amplified by PCR and detected by agarose gel electrophoresis. In the experiment, we have tried our best to avoid any cross-contamination of pork DNA between the samples during all the preparation steps. The results revealed that only 3 samples which
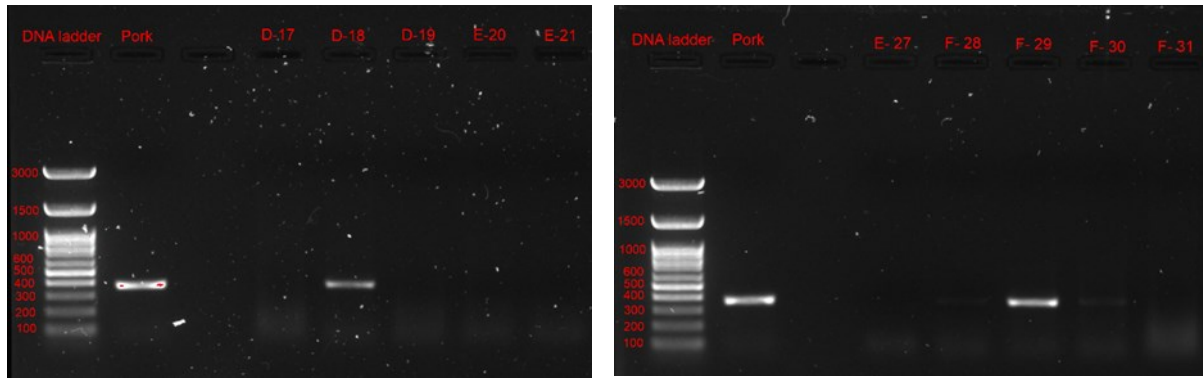
Figure 2. Agarose gel electrophoresis of PCR products of the initial screening step. Samples of D-18 and F-29 showed a thick and clear specific single band of size 387 bp.

Table 2. Detection of pork adulteration in processed meat samples by PCR. '+' indicates the presence of pork and '-' indicates the absence of pork.

Source	Product category	Sample name	Presence/ absence of pork
Stall 1	Sausage	A-1	-
Stall 1	Sausage	A-2	-
Stall 1	Sausage	A-3	-
Stall 1	Sausage	A-4	-
Stall 1	Patties	A-5	-
Stall 2	Sausage	B-6	+
Stall 2	Sausage	B-7	+
Stall 2	Sate	B-8	+
Stall 2	Fish ball	B-9	-
Stall 2	Sausage	B-10	+
Stall 2	Sate	B-11	-
Stall 3	Patties	C-12	-
Stall 3	Sausage	C-13	-
Stall 3	Sate	C-14	-
Stall 3	Sate	C-15	-
Stall 4	Sausage	D-16	-
Stall 4	Sausage	D-17	-
Stall 4	Meat/chicken ball	D-18	+
Stall 4	Sausage/patties	D-19	-
Stall 5	Meat/chicken ball	E-20	-
Stall 5	Meat/chicken ball	E-21	-
Stall 5	Sausage	E-22	-
Stall 5	Sausage	E-23	-
Stall 5	Sausage	E-24	-
Stall 5	Patties	E-25	-
Stall 5	Meat/fish ball	E-26	-
Stall 5	Meat/fish ball	E-27	-
Stall 6	Sate	F-28	+
Stall 6	Sausage	F-29	+
Stall 6	Sate	F-30	+
Stall 6	Sate	F-31	-
Stall 7	Sausage	G-32	-
Stall 7	Sausage	G-33	-
Stall 7	Sausage	G-34	-
Stall 7	Meat/chicken ball	G-35	-
Stall 7	Patties	G-36	-

were D-18, F-29 and F-30 were positively identified to produce the specific single band of size 387 bp (Figure 3). Agarose gel electrophoresis image indicated that samples of D-18 and F-29 produced a clear and specific single band of the expected size of pork DNA. For the F-30 sample, the detectable band was quite faint. The validation results indicated that the processed meat samples of D-18, F-29 and F-30 likely to contain pork DNA which consistent with the initial screening results.
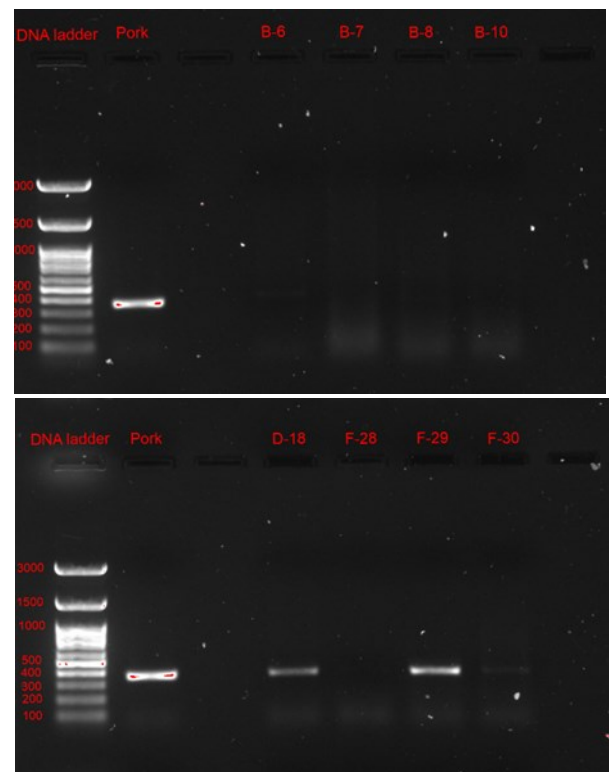

Figure 3. Validation of agarose gel electrophoresis of PCR products of 8 samples from the initial screening results. Samples D-18, F-29 and F-30 show single band of size 387 bp.

The absence of a single band of pork DNA in the 5 samples, B-6, B-7, B-8, B-10, and F-28 of processed meat products in the validation results could be due to several reasons. Compared to the validation result, the presence of bands in the screening step might be due to cross contamination happens between the samples from the same stall as this could be true for the samples of B-6, B-7, B-8, B-10, and F-28, F-29, F-30, respectively. In

addition, we expect that the different portion or location of the samples taken for the DNA extraction between the initial screening and validation steps likely to cause the difference in results. The samples taken from the portions that were collected during the validation possibly did not contain pork in contrast to the location of samples taken in the initial screening experiment. With all the results presented, the presence of a single and specific band in the 3 samples, D-18, F-29, and F-30 give conclusive evidence of the presence of pork in the samples. Cross-contamination of meats may happen in the meat processing stage particularly during the meat grinding operation (Doosti *et al*., 2014). When the same grinder is used, the routine practice is to clean the grinder after processing different types of meats but this is always not happened. Furthermore, as pork is cheaper compared to beef, the many cases of meat adulterated with pork are primarily driven by this economic reason (Hahn, 2004).

The presence of many Muslim street food stalls selling various types of processed meat products in Hat Yai indicating a good demand of those kinds of food products from consumers. Nevertheless, as halal certification is voluntarily and no legal requirement for Muslims' street stalls or restaurants to properly display authorized halal label, all the premises are just self-proclaimed as "halal". The confidence of Muslim consumers is solely based on the trust towards the "halal brand", thus this will definitely open the many possible ways for the occurrence of food adulteration. In this study, the detection of pork DNA in the samples collected indicates the presence of pork in processed meat products. The result highlights the importance of awareness and knowledge on halal particularly among the Muslim business owners to ensure the products that they are selling are halal from its origin and sources. The halal integrity of the entities involved in the food supply chain, in particular, the suppliers or producers should be transparent to give confidence to the Muslim consumers about the halal foods.

4. Conclusion

The present results of the study reveal the real cases of halal food adulteration with pork in processed meat products from "halal" labelled Muslim food stalls. The results are significantly useful for authorities that govern the halal related matters in Thailand. The effort to ensure halal integrity is kept preserved for halal food products requires full commitment and cooperation from each layer of the supply chain. Notably, as the tourism sector is one of the important driving economic growth of Hat Yai, therefore proactive actions from the government and halal authorities of Thailand are greatly significant to cater on the problem of food adulteration.

Conflict of Interest

The authors declare no conflict of interest.

References

Aida, A.A., Che Man, Y.B., Wong, C.M.V.L., Raha, A.R. and Son, R. (2005). Analysis of raw meats and fats of pigs using polymerase chain reaction for halal authentication. *Meat Science*, 69(1), 47–52. https://doi.org/10.1016/j.meatsci.2004.06.020

Chai, Y., He, Y., Lv, R., Chen, H., Wang, Q. and Pan, L. (2017). Detection and quantification of beef and pork materials in meat products by duplex droplet digital PCR. *PLoS ONE*, 12(8), e0181949. https://doi.org/10.1371/journal.pone.0181949

Che Man, Y.B., Aida, A.A., Raha, A.R. and Son, R. (2007). Identification of pork derivatives in food products by species-species polymerase chain reaction (PCR) for halal verification. *Food Control*, 18(7), 885–889. https://doi.org/10.1016/j.foodcont.2006.05.004

Calvo, J.H., Osta, R. and Zaragoza, P. (2002). Quantitative PCR detection of pork in raw and heated ground beef and Pâté. *Journal of Agricultural and Food Chemistry*, 50(19), 5265-5267. https://doi.org/10.1021/jf0201576

Doosti, A., Dehkordi, P.G. and Rahimi, E. (2014). Molecular assay to fraud identification of meat products. *Journal of Food Science and Technology*, 51(1), 148–152. https://doi.org/10.1007/s13197-011-0456-3

Di Pinto, A., Bottaro, M., Bonerba, E., Bozzo, G., Ceci, E., Marchetti, P., Mottola, A. and Tantillo, G. (2015). Occurrence of mislabeling in meat products using DNA-based assay. *Journal of Food Science and Technology,* 52(4), 2479–2484. https://doi.org/10.1007/s13197-014-1552-y

Fadzlillah, N.A., Che Man, Y.B. and Jamaludin, M.A. (2011). Halal food issues from Islamic and modern science perspective. *2011 2nd International Conference on Humanities, Historical and Social Sciences (IPEDR),* 17, 159-163.

Ha, J., Kim, S., Lee, J., Lee, S., Lee, H., Choi, Y., Oh, H. and Yoon, Y. (2017). Identification of pork adulteration in processed meat products using the developed mitochondrial DNA-based primers. *Korean Journal for Food Science of Animal Resources*, 37(3), 464-468. https://doi.org/10.5851/kosfa.2017.37.3.464

Hahn W. (2004). Beef and pork values and price spreads

explained. Livestock, Dairy and Poultry Outlook No: LDP-M-118-01., p. 30. USA: USDA, Economic Research Service.

Kuncharin, W. and Mohamed, B. (2013). Cross-border shopping motivation, behaviours and ethnocentrism of Malaysian in Hat Yai, Thailand. *International Journal of Social, Behavioral, Educational, Economic, Business and Industrial Engineering*, 7 (4), 1017-1027.

Karabasanavar, N.S., Singh, S.P, Kumar, D. and Shebannavar, S.N. (2014). Detection of pork adulteration by highly-specific PCR assay of mitochondrial D-loop. *Food Chemistry*, 145, 530-534. https://doi.org/10.1016/j.foodchem.2013.08.084

Mohayidin, M.G. and Kamarulzaman, N.H. (2014). Consumers' preferences toward attributes of manufactured halal food products. *Journal of International Food and Agribusiness Marketing*, 26 (2), 125-139. https://doi.org/10.1080/08974438.2012.755720

Montiel-Sosa, J.F., Ruiz-Pesini, E., Montoya, J., Roncalés, P., López-Pérez, M.J. and Pérez-Martos (2000). A Direct and highly species-specific detection of pork meat and fat in meat products by PCR amplification of mitochondrial DNA. *Journal of Agricultural and Food Chemistry*, 48(7), 2829-2832. https://doi.org/10.1021/jf9907438

Ruslan, A.A.A., Kamarulzaman, N.H. and Sanny, M. (2018). Muslim consumers' awareness and perception of halal food fraud. *International Food Research Journal*, 25(Suppl. 1), S87-S96.

Rahmati, S., Julkapli, N.M., Yehye, W.A. and Basirun, W.J. (2016). Identification of meat origin in food products - A review. *Food Control*, 68, 379-390. https://doi.org/10.1016/j.foodcont.2016.04.013

Rodríguez, M.A., García, T., González, I., Asensio, L., Mayoral, B., López-Calleja, I., Hernández, P.E., Martín, R. (2003). Identification of goose, mule duck, chicken, turkey and swine in foie gras by species-specific polymerase chain reaction. *Journal of Agricultural and Food Chemistry*, 51(6), 1524-1529. https://doi.org/10.1021/jf025784+

Shaari, J.A.N. and Ariffin, N.S.M. (2010). Dimension of halal purchase intention: A preliminary study. *International Review of Business Research Papers*, 6 (4), 444–456.