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Market surveillance of porcine DNA detection in commercial food products in Sibu, Sarawak

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Abstract

Porcine adulteration in food products is unacceptable to consumers who avoid pork consumption due to religious or health reasons; hence, detecting pork and its derivatives in food products is vital. The present work focused on assessing the DNA extraction efficiency of salt method as compared to the DNeasy mericon Food Kit to detect porcine DNA *via* quantitative PCR (qPCR), and comparing these qPCR findings with the food product labelling. The study selected food products which lacked JAKIM (Jabatan Kemajuan Islam Malaysia) certified halal logo, and those bearing foreign or counterfeit halal logos in Sibu, Sarawak. Twenty-four (n = 24) commercial food products, three (n = 3) pork-based products (positive control), and three (n = 3) JAKIM halal-certified products (negative control) were included. DNA was isolated and used as a template in a qPCR assay to target cytochrome b (*cytb*). Positive samples were sent for DNA sequencing. The experimental output was compared with the food ingredient and presence of a halal logo on product labelling. Out of 30 samples extracted using the DNeasy mericon Food Kit, DNA from all samples (100%) fell within the optimal DNA purity ratio which ranged from 1.7 to 2.0. The DNA extracted using this method was further used as a template in the qPCR. The qPCR assay demonstrated presence of porcine DNA in two food samples which lacked product labelling, with mean C_t values ± SD of 19.05 ± 0.72 and 28.07 ± 1.67 as compared to the positive control (mean C_t values ± SD of 13.44 ± 0.37 to 14.78 ± 1.10). Basic Local Alignment Search Tool (BLAST) analysis revealed a high percentage identity (94.74 - 100%) to *Sus scrofa domesticus* (pig) as compared to sequences in the National Centre for Biotechnology Information (NCBI) database. The present work demonstrated a significant halal status of various food items for Muslims and individuals with pork allergies in the studied area.

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Introduction

Food adulteration has raised public concern particularly amongst consumers. Food adulteration refers to intentional lowering of food quality by adding, substituting with cheaper or inferior ingredients, or removing valuable components (Banti, 2020). Consumers expressed concern over food adulteration due to its typically negative implications from economic, health, religious, or legal standpoints. In certain retail environments, it is commonplace to find meat products adulterated by

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replacing higher-value meats with lower-value or quality. This practice is typically carried out to decrease the cost of a food product or to augment its total volume. For instance, the substitution of expensive beef with pork in product formulation due to cheaper, readily available, and has a similar shape and colour to the beef (Novianty *et al*., 2017). However, this is completely unacceptable to consumers who avoid the consumption of pork for ethical, religious, and health reasons (Ali *et al*., 2012).

Besides, incidents of food product mislabelling are on the rise. Mislabelling occurs when the label of a food product fails to correctly represent its contents (AIFS, 2022). Such instances of mislabelling lead to a decline in consumer confidence in the food supply chains and regulatory bodies. Amongst many types of commercial food sample, meat and meat-based products are often the targets of mislabelling incidents due to their high demand for consumption (Perestam *et al*., 2017). Identifying pork in processed meat products is harder through visual examination than in fresh unprocessed meats. Processing methods alter the appearance, colour, texture, and flavour of meat, enabling manufacturers to easily disguise the source and origin of ingredients in meat mixtures. Furthermore, food processing techniques such as grinding and mixing meat alter the produced meat products (Esteki *et al*., 2019). Consequently, the issue has become a major concern not only from an economic standpoint, but also from a religious perspective, especially when the product contains pork, but it is not indicated on the label.

Fraudulent adulteration and mislabelling of food products with undeclared meat components also affect individuals who are prone to allergic reactions or sensitised individuals restricted by certain diet regimes, such as vegetarians. Increasing evidence suggests that meat can trigger allergic responses in sensitive individuals. In 2009, the Food Standards Agency (FSA) in the UK found that various companies were injecting altered bulking agents made from pork and beef products into chicken products to retain water and increase weight. Consequently, an individual who is allergic to pork may experience an adverse immune response after consuming pork-adulterated chicken (Everstine *et al*., 2013). Skin rashes, nausea, vomiting, breathing difficulty, and mild fever are signs of pork allergy (NYASU, 2022).

Islam ranks as the world's second most followed religion, after Christianity, with a global Muslim population that exceeds two billion (World Population Review, 2022). Malaysia is home to multi-ethnicity, and amongst the religions embraced are Islam, Christianity, and Buddhism. In 2020, Malaysia's Muslim population stood at 20.6 million, making up 63.5% of the country's total population (DOSM, 2022). With the increasing Muslim population, halal-certified foods have emerged as a significant market segment. Accounting for over 20% of the global food industry today, the halal food market is expected to constitute 70% by 2050 (Ruslan *et al*., 2018).

Halal certification not only indicates that the food is free from non-halal ingredients, but also indicates the wholesomeness of the food. Owing to motivation to gain profit by irresponsible manufacturers, the misuse of halal certification is alarming amongst the Muslim community. Halal logo misconduct had been reported, involving a frozen meat company in Johor Bahru, whereby fake halal logos were used on their products (Tan, 2021). Similarly, a case of halal logo misconduct was reported in Johor, whereby a frozen meat company in Senai Industrial Park used counterfeit halal labels and stamps. These were applied to packaged meat, and distributed across the country (Rahim *et al*., 2020). Consequently, consumers have lost faith in these products, and are less likely to buy those that were once certified as halal.

In recent years, there was a significant rise in the level of concern within the food sector in regard to halal authenticity of various food products. Currently, most halal products in Malaysia’s local markets are manufactured by non-Muslim producers. However, most non-Muslims do not fully comprehend the implications of Islamic dietary laws (Ruslan *et al*., 2018). Insufficient knowledge, awareness, and understanding of halal principles within the scope of halal product manufacturing could potentially result in deterioration of ethical and religious principles that are linked to halal practices.

Furthermore, instances of fraudulent activities within the halal food sector persist as certain entities, such as companies, proprietors, and manufacturers, fail to comply with the Shariah law. These non-halal compliances involve incorporation of non-halal ingredients into food products, deceptive use of counterfeit halal certifications, adulteration of halal food with non-halal components, and the utilisation of non-halal distribution and marketing channels for their product handling (Ruslan *et al*., 2018). These

issues may create an unpleasant feeling amongst Muslims. Therefore, the manufacturers are responsible for ensuring that their raw ingredients and additives are from halal sources so that the halal integrity of food products is not compromised (Mohd Hafidz *et al*., 2020).

Owing to the prevalent challenge of food adulteration and importance to uphold integrity of halal products, a range of analytical techniques were developed to precisely and sensitively identify pork in meat products. Two important methodologies employed in this context are protein-based and DNA-based analyses, which are instrumental in detecting pork and its derivatives in foods. The utilisation of protein-centric analytical approaches is generally constrained to extensively processed meat products, as these products frequently undergo severe processing conditions, including elevated temperatures and substantial pressure that can easily denature proteins; hence, rendering protein analysis less reliable for pork detection.

In contrast, DNA has been recognised for its stability and ability to withstand extreme temperatures and pressures associated with meat processing (Mohd Hafidz *et al*., 2020). Therefore, DNA-based methods could be more viable for identifying DNA in processed meat samples, offering a more robust, straightforward, and rapid approach for the detection of pork and its by-products. Although conventional PCR is a straightforward, user-friendly, and widely adopted DNA-based method for identifying species-specific DNA in food products, its requirement for endpoint analysis renders it a time-intensive method. Moreover, conventional PCR cannot provide quantitative information about the targets that are initially present in the sample. Alternatively, quantitative real-time polymerase chain reaction (qPCR), which uses specially designed primers and probes to accurately identify trace amounts of DNA (Erwanto *et al*., 2018), is inherently more accurate, specific, sensitive, less time-consuming, and less laborious than conventional PCR (Ali *et al*., 2012).

Due to scarcity of information regarding the mislabelling or adulteration of porcine DNA amongst various food groups in Sarawak, particularly in Sibu, the present work could provide preliminary information to the target community. In addition, studies on laboratory testing for the detection of porcine DNA collected from food production systems are lacking. Therefore, the present work would shed some light on the importance of laboratory testing for porcine DNA detection, thereby providing essential insights into the halal status of food products within the target community. Accordingly, the present work was undertaken to assess the presence of porcine DNA in a diverse array of food products available in Sibu, Sarawak, using a qPCR assay, and comparing the outcome of the result with food labelling, especially the ingredients and presence of halal logo.

Materials and methods

Samples

Twenty-four (n = 24) commercial food products were acquired from various supermarkets and a local restaurant in Sibu, Sarawak which included frozen meat products (n = 10), seasonings (n = 2), and canned meat products (n = 12). Food products were collected based on the following criteria: lack of the certified halal logo as accredited by JAKIM, presence of fake halal logos, foreign halal logos, and absence of a proper labelling statement. Table 1 lists the food products analysed in the present work.

Primer and probe sequences

A set of primers and TaqMan probe sequences which specifically targeted the *cytb* mitochondrial region, as designed by Sajali *et al*. (2022), were employed as detection markers. The primer pairs (F: CAAAGCAACCCTCACACGAT and R: AGATTCCGGTAGGGTTGTTG) and 20-nt porcine TaqMan probe (5HEX-TTACCGCCCTCGCAGCCGTA-3IABkFQ) were designed to amplify a 121-base pair (bp) segment of the *Sus scrofa domesticus* mitochondrial *cytb*. The TaqMan probe was designed by tagging 5-hexachlorofluoresceine (5-HEX) and 3-Iowa black FQ (3-IABkFQ) at the 5′ and 3′ ends, respectively. These primers and probe sequences were validated using the BLAST software available at the NCBI to confirm their specificity. The primers and probes were synthesised by Integrated DNA Technologies (Singapore).

DNA extraction by DNeasy mericon Food Kit

Genomic material (DNA) was extracted from the commercial food products using a DNeasy mericon Food Kit, (Qiagen, DEU) in accordance with

Table 1. Samples tested and control samples.

No.	Code	Product	Type
1.	C01	Frozen honey chicken chop	Frozen food
2.	C02	*Char siu* chicken chop	Frozen food
3.	C03	Sticky rice with *pandan* chicken	Frozen food
4.	C04	Cabbage chicken dumpling	Frozen food
5.	C05	*Pandan* chicken	Frozen food
6.	C06	Chicken burger	Frozen food
7.	C07	Taro chicken dumpling	Frozen food
8.	B01	Beef burger	Frozen food
9.	B02	Beef meatball	Frozen food
10.	F03	Mackerel fishball	Frozen food
11.	S01	*Pansuh* paste	Condiment
12.	S03	*Kacangma* paste	Condiment
13.	DF1	Fried dace with salted black bean 1	Canned food
14.	DF2	Fried dace with salted black bean (premium) 2	Canned food
15.	DF3	Fried dace with salted black bean 3	Canned food
16.	DF4	Fried dace with bean curd stick (premium) 4	Canned food
17.	DF5	Fried dace with salted black bean 5	Canned food
18.	DF6	Fried dace with salted black bean 6	Canned food
19.	S1	Spicy sardine	Canned food
20.	S2	Sardine in brine	Canned food
21.	S3	Fried sardine and salted black bean in soy sauce	Canned food
22.	S4	Fried sardine in chilli sauce	Canned food
23.	S5	Sardine in tomato sauce 1	Canned food
24.	S6	Sardine in tomato sauce 2	Canned food
		Positive control	
1.	P01	Pork meatball	Frozen food
2.	P02	Pork salami	Frozen food
3.	P03	Smoked back bacon	Frozen food
		Negative control	
1.	N01	Streaky beef breakfast slice	Frozen food
2.	N02	Chicken meatball	Frozen food
3.	N03	Beef burger	Frozen food

the manufacturer's protocols. Specifically, 200 mg of the food samples were carefully weighed and transferred into a microcentrifuge tube, followed by the addition of 1 mL of food lysis buffer and 2.5 µL of proteinase K solution. The mixture was briefly vortexed to ensure complete distribution and moistening of sample material. The mixture was incubated in a thermomixer for 30 min at 60°C with constant shaking (1,000 rpm). At the end of incubation, the solution was cooled to room temperature (15 - 25°C) on ice to enhance inhibitor precipitation. The samples were centrifuged at 2,500 *g* for 5 min. Subsequently, 500 µL of chloroform was dispensed into a new microcentrifuge tube. Following centrifugation, utmost care was taken to aspirate 700 µL of clear supernatant into a microcentrifuge tube that contained chloroform, without disturbing the inhibitor precipitate at bottom of the tube. The microcentrifuge tube that contained chloroform was subsequently vortexed vigorously for 15 s, and subjected to centrifugation at 14,000 *g* for 15 min. This centrifugation process resulted in the formation of three distinct layers within the tube: an upper aqueous phase, an interphase, and a lower organic phase. Then, 1 mL of PB buffer was pipetted into a new microcentrifuge tube, and 250 µL of the upper

aqueous phase was added and thoroughly mixed by vortexing.

Next, 600 µL of mixture from the microcentrifuge tube was carefully pipetted into the QIAquick spin column, which was then placed inside a 2 mL collection tube. The QIAquick spin column was centrifuged at 17,900 *g* for 1 min, and the resulted flow-through was discarded. This step was repeated to ensure that complete binding of DNA to the spin column, and any flow-through that remained was discarded. Following this, 500 µL of AW2 buffer was added into the QIAquick spin column, which was subsequently subjected to centrifugation at 17,900 *g* for 1 min, and the flow-through was discarded. The centrifugation step was repeated to ensure that the membrane was thoroughly dried. Then, the QIAquick spin column was carefully transferred into a new microcentrifuge tube. To elute the DNA, 100 µL of EB buffer was pipetted directly onto the QIAquick membrane. The QIAquick membrane was incubated for 1 min at room temperature (15 - 25°C), and then centrifuged at 17,900 *g* for 1 min. This step effectively facilitated the elution of DNA from the column. The extracted DNA was subsequently stored at -20°C until further use.

DNA extraction by salt method

DNA extraction by the salt method used in this study was according to the procedure previously reported by Yalçınkaya *et al*. (2017). Fifty milligrams of each homogenised sample was accurately weighed and deposited into individual microcentrifuge tubes, and subsequently solubilised in a solution containing 400 µL of lysis buffer (containing 10 mM Tris-HCl, pH 8.0; 2 mM EDTA, pH 8.0; and 0.4 M NaCl) along with 40 µL of 20% (w/v) SDS. The mixtures were thoroughly mixed by vortexing, ensuring proper homogenisation. To this mixture, 2 µL of 20 mg/mL Proteinase K (Vivantis, Malaysia) was added, and the mixture was incubated at 65°C for 1 h. Following incubation, 300 µL of 6 M NaCl was added, and the mixture was again vortexed vigorously for 30 s. After a subsequent centrifugation step at 10,000 *g* for 30 min, the supernatant was carefully transferred into a new microcentrifuge tube. Equal volumes of isopropanol were added to the supernatant, and the resulted mixture was vortexed before incubated at 20°C for 10 min. Then, the mixture was centrifuged at 16,000 *g* for 20 min to separate the supernatant from pellet. The supernatant was discarded, and the resulted pellet was dried. The dried pellet was subsequently dissolved in 100 µL of TE buffer.

Determination of DNA concentration and purity

DNA purity was assessed using a Cary 60 UV-Vis Spectrophotometer (Agilent, USA). The spectrophotometer was calibrated with a blank solution which contained 700 µL of Tris-HCl prior to analysis. Then, the DNA sample was diluted 100-fold by adding 693 µL of Tris-HCl to 7 µL of DNA sample. Absorbance of the diluted DNA sample was subsequently recorded at wavelengths of 260 nm (A_{260}) and 280 nm (A_{280}). DNA purity was calculated as ratio of absorbance from 260 to 280 nm (A_{260}/A_{280}). To determine the DNA concentration, Eq. 1 was used, as previously described by Barbas III *et al*. (2007):

$$\text{DNA concentration} = 50\ \mu g/mL \times A_{260} \times DF \quad \text{(Eq. 1)}$$

where, A_{260} of 1.0 = 50 µg/mL pure double-stranded DNA (constant value); A_{260} = absorbance reading at 260 nm; and DF = dilution factor.

Determination of DNA integrity

To evaluate DNA integrity, 1% (w/v) of agarose gel electrophoresis was used. Initially, 400 mg of agarose powder (Vivantis, USA) was accurately weighed and subsequently dissolved in 40 mL of 1× TBE Buffer (Vivantis, USA). Then, the mixture was gently heated in a microwave oven for a few minutes until the agarose was completely dissolved. Consequently, the molten agarose solution was allowed to cool under running tap water. Then, 1 µL of SYBR Safe DNA stain (Invitrogen, USA) was added to the cooled molten agarose solution. Stained molten agarose solution was carefully poured into a gel mould with the gel comb properly positioned, and the mixture was allowed to solidify at room temperature for approximately 20 min. After the agarose solidified, the gel comb was carefully removed.

The agarose gel was subsequently placed in a gel chamber that was filled with 1× TBE buffer. To validate the quality and preparation of the agarose gel, 1 µL of 1 kb DNA ladder (Promega, USA) was loaded onto the first lane. For each DNA sample under examination, a mixture was prepared by mixing 3 µL of the DNA samples with 1 µL of 6× DNA loading dye (Fermantas, USA). The mixture was then loaded

into individual wells on the agarose gel. Gel electrophoresis was conducted at 120 V for 30 min. Following electrophoresis, the resulted gel image was visualised using a gel imager.

Construction of qPCR standard curve

To assess amplification efficiency of the primer pair and probe sequences in qPCR, a standard curve was established following the method outlined by Sajali *et al*. (2022). Porcine DNA was subjected to five 10-fold serial dilutions (ranging from 10^{-1} to 10^{-5}) to generate the standard curve. The master mix was prepared in a 1.5 mL microcentrifuge tube, containing the total number of reactions required for each experiment. The master mix was then dispensed into individual PCR tubes, each containing 5 µL of 2× GoTaq Probe qPCR Master Mix (Promega, USA), 0.25 µL of forward and reverse primers (0.25 µM each), 0.125 µL of hydrolysis TaqMan probe (0.125 µM), and 3.375 µL of nuclease-free water. Finally, 1 µL of the DNA template was added to each corresponding PCR tube, which resulted in a final reaction volume of 10 µL.

For each experimental run, five tubes containing DNA templates with 10-fold serial dilutions were concurrently conducted in triplicates. Reaction mixtures without the template or no template control (NTC) were incorporated as negative control. Following preparation of the reaction mixtures, the PCR tubes were briefly spun and then loaded into a DTprime Real-time thermal cycler (DNA-Technology, Russia). The thermal cycling conditions were as follows: initial cycle for GoTaq® DNA polymerase activation at 95°C for 2 min, followed by 40 cycles which involved denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min.

The standard curve was constructed by plotting the threshold cycle (C_t) against the logarithmically transformed DNA concentration. Efficiency and linearity were determined using correlation coefficient (R^2) of the qPCR assay derived from the standard curve. Mean values and standard curves between each repetition were analysed. To assess the amplification efficiency of the qPCR assay, Eq. 2 (Bio-Rad, 2006) was employed as follows:

$$E = 10^{\frac{-1}{slope}} - 1 \qquad \text{(Eq. 2)}$$

where, E = theoretical efficiency; and slope = 'm' in $y = mx + c$.

The amplification efficiency of qPCR was also expressed as a percentage, providing insights into the proportion of template amplified during each qPCR cycle. An efficiency of 100% corresponded to a slope of -3.32. The range of acceptable slope values, from -3.1 to -3.58, signified qPCR efficiencies between 90 and 110%. To convert the amplification efficiency into percentage, Eq. 3, as outlined by Bio-Rad (2006), was applied:

$$\%\, Efficiency = (E - 1) \times 100\% \qquad \text{(Eq. 3)}$$

where, E = theoretical efficiency.

Detection of porcine DNA using qPCR

The protocol for conducting qPCR and thermal cycling conditions were primarily based on the product information provided by GoTaq Probe qPCR Master Mix (Promega, USA), with a slight modification made to the total reaction volume as required for the specific experiment. The master mix was prepared in a 1.5 mL microcentrifuge tube, then dispensed into individual PCR tubes, each containing 5 µL of 2× GoTaq Probe qPCR Master Mix (Promega, USA), 0.25 µL of forward and reverse primers (0.25 µM each), 0.125 µL of hydrolysis TaqMan probe (0.125 µM), and 3.375 µL of nuclease-free water. Finally, 1 µL of the DNA template was added to each corresponding PCR tube, resulting in a final reaction volume of 10 µL.

Porcine DNA extracted from commercial pork products was used as a positive control, while DNA extracted from samples with a JAKIM halal-certified logo served as a negative control. Both positive and negative controls were integrated into the experimental setup to ensure reliability and validity of the experimental data. Additionally, NTC was prepared by substituting DNA with nuclease-free water. The PCR tubes were capped and briefly centrifuged to ensure a consistent reagent distribution, and all reagent components remained at the bottom of tubes. Following this step, the PCR tubes were loaded into a DTprime Real-time thermal cycler (DNA Technology, Russia), and the experiments were conducted in triplicates.

Validation of positive samples

For DNA sequencing, samples that contained porcine DNA and identified as positive samples were prepared. The qPCR reactions were performed with a slight modification to the total reaction volume,

which was set at 40 µL per reaction. The qPCR assay was carried out following the recommended thermal cycling conditions, as detailed earlier. The qPCR products were subsequently sent to Apical Scientific Sdn. Bhd. in Selangor for comprehensive sequencing analysis. The resulting DNA sequencing data were subjected to analysis using BLAST software, which was accessible through NCBI.

Results and discussion

Validation of primer and probe sequences specificity

The effectiveness of qPCR relies heavily on the selection of suitable primer and probe sequences. These sequences must be sufficiently sensitive and specific to detect the desired target species. Specificity of primer and probe sequences signify the distinct identity of the species (Kralik and Ricchi, 2017). In the present work, the primers and probe sequences designed by Sajali *et al*. (2022) were employed. To validate their specificity to the target species *Sus scrofa domesticus*, these sequences were subjected to verification using BLAST in the NCBI database. This step ensured that the selected sequences specifically targeted the intended species.

BLAST analysis of the query sequence for the forward primer, reverse primer, and probe sequences demonstrated 100% match with *Sus scrofa domesticus* in the NCBI database. Therefore, the primer pair and probe used in the present work demonstrated high specificity which exclusively amplified the intended target of *Sus scrofa domesticus*, with no detection of unintended targets. This specificity was further confirmed through the experimental process, ensuring the precision of the qPCR assay. Similarly, the probe sequence generated a signal only when bound to the targeted sequence of *Sus scrofa domesticus*. High specificity was crucial for qPCR assays, whereby quantification relied on the total intensity of fluorescence in the amplified DNA. The unintended target amplification could significantly affect accuracy. Ensuring specific amplification of *Sus scrofa domesticus* was thus vital for reproducibility and precision.

Determination of DNA purity by UV-Visible (UV-Vis) spectrophotometry

Reliable measurement of DNA purity is important for several applications in molecular biology because it determines the success or failure of downstream analyses. Comprehensive assessment of DNA quality entails a combination of UV-Vis spectrophotometric measurements and agarose gel electrophoresis. To determine DNA purity, absorbance readings at 260 nm (A_{260}) and 280 nm (A_{280}) were measured using a UV-Vis spectrophotometer in a quartz cuvette. The ratio of these readings at 260 and 280 nm (A_{260}/A_{280}) offered an indication of DNA purity. Table 2 presents the A_{260}/A_{280} ratios for each food sample's DNA using the DNeasy mericon Food Kit and salt method. Additionally, the mean C_t values obtained from qPCR of these samples was documented for two independent studies.

DNA was considered pure when the A_{260}/A_{280} ratios fell within the specified absorbance range of 1.7 - 2.0 (Oswald, 2016). Figure 1 illustrates a comparison of the absorbance ratios of food samples extracted using both the DNeasy mericon Food Kit and salt method. In particular, all DNA extracted from food samples (100%) using the DNeasy mericon Food Kit was within the optimal range of absorbance ratios, with none of the DNA samples (0%) fell outside the optimal absorbance ratio range (less than 1.7 and above 2.0). In contrast to the DNeasy mericon Food Kit method, the results indicated that nine out of 30 DNA samples (30%) had an absorbance ratio of below 1.7, while 12 out of 30 DNA samples (40%) fell within the optimal absorbance ratio range, and nine out of 30 DNA samples (30%) exhibited an absorbance ratio which exceeded 2.0. Consequently, it could be concluded that the DNeasy mericon Food Kit was a more favourable choice for DNA extraction as it consistently yielded high-purity and high-quality DNA as compared to the salt method.

The A_{260}/A_{280} ratio of below 1.7 indicated that the samples were either contaminated by protein or chemical reagents associated with the extraction protocol. Residual impurities that persisted from the DNA extraction procedure, including substances like ethanol, chloroform, or phenol, could introduce interference in spectrophotometric analysis, leading to a decrease in the A_{260}/A_{280} ratio of extracted DNA. Additionally, incomplete cell lysis during the DNA extraction process could also result in diminished DNA yield, primarily due to the inability to fully disrupt the cell membrane of food sample.

In the present work, daces and sardines that were extracted using the salt method demonstrated protein contamination. This could be attributed to the

Table 2. Mean absorbance ratio (A_{260}/A_{280}) of food samples' DNA extracted using DNeasy mericon Food Kit, salt method, and mean C_t ± SD values of food samples' DNA detected using qPCR.

No.	Code	Products	Type of food products	Declared meat species	Mean ± SD		Mean C_t ± SD	Halal symbol
					DNeasy mericon Food Kit	Salt method		
1.	C01	Frozen honey chicken chop	Frozen food	Chicken	2.03 ± 0.04	1.82 ± 0.29	40 ± 0.00*	NA
2.	C02	*Char siu* chicken chop	Frozen food	Chicken	1.96 ± 0.02	1.99 ± 0.03	40 ± 0.00	NA
3.	C03	Sticky rice with *pandan* chicken	Frozen food	NA	1.93 ± 0.04	1.98 ± 0.01	36.23 ± 4.22	NA
4.	C04	Cabbage chicken dumpling	Frozen food	Chicken	2.02 ± 0.06	2.03 ± 0.00	37.38 ± 4.19	NA
5.	C05	*Pandan* chicken	Frozen food	Chicken	1.99 ± 0.09	2.06 ± 0.07	40 ± 0.00	NA
6.	C06	Chicken burger	Frozen food	Chicken	2.05 ± 0.01	2.11 ± 0.01	40 ± 0.00	NA
7.	C07	Taro chicken dumpling	Frozen food	NA	1.99 ± 0.01	2.04 ± 0.03	19.05 ± 0.72	NA
8.	B01	Beef burger	Frozen food	Beef	1.98 ± 0.03	2.06 ± 0.00	40 ± 0.00	NA
9.	B02	Beef meatball	Frozen food	NA	1.91 ± 0.11	2.03 ± 0.09	40 ± 0.00	NA
10.	F03	Mackerel fishball	Frozen food	NA	1.97 ± 0.03	1.90 ± 0.02	28.07 ± 1.67	NA
11.	S01	*Pansuh* paste	Condiment	Chicken flavour	2.03 ± 0.12	2.34 ± 0.22	37.72 ± 3.54	NA
12.	S03	*Kacangma* paste	Condiment	Chicken stock	1.71 ± 0.17	2.10 ± 0.05	38.77 ± 3.02	NA
13.	DF1	Fried dace with salted black bean 1	Canned food	Dace	1.84 ± 0.00	1.50 ± 0.16	40 ± 0.00	√
14.	DF2	Fried dace with salted black bean (premium) 2	Canned food	Dace	1.80 ± 0.07	1.71 ± 0.07	38.88 ± 2.74	NA
15.	DF3	Fried dace with salted black bean 3	Canned food	Dace (fish)	1.99 ± 0.02	1.68 ± 0.06	40 ± 0.00	√
16.	DF4	Fried dace with bean curd stick (premium) 4	Canned food	Dace	1.91 ± 0.03	1.40 ± 0.08	40 ± 0.00	NA
17.	DF5	Fried dace with salted black bean 5	Canned food	Dace	1.83 ± 0.16	1.54 ± 0.03	40 ± 0.00	NA
18.	DF6	Fried dace with salted black bean 6	Canned food	Dace (fish)	1.91 ± 0.11	1.82 ± 0.22	38.67 ± 3.27	√
19.	S1	Spicy sardine	Canned food	Sardines	1.88 ± 0.05	1.73 ± 0.15	40 ± 0.00	NA
20.	S2	Sardine in brine	Canned food	Sardines	1.78 ± 0.06	1.50 ± 0.19	40 ± 0.00	NA
21.	S3	Fried sardine and salted black bean in soy sauce	Canned food	Sardines	1.87 ± 0.04	1.65 ± 0.04	40 ± 0.00	NA
22.	S4	Fried sardine in chilli sauce	Canned food	Sardines	1.93 ± 0.04	1.77 ± 0.10	40 ± 0.00	NA
23.	S5	Sardine in tomato sauce 1	Canned food	Sardines	1.78 ± 0.19	1.49 ± 0.13	40 ± 0.00	√
24.	S6	Sardine in tomato sauce 2	Canned food	Sardines	1.86 ± 0.07	1.59 ± 0.18	39.03 ± 2.37	√
		Positive control						
1.	P01	Pork meatball	Frozen food	Pork	2.07 ± 0.10	1.95 ± 0.08	13.85 ± 0.38	NA
2.	P02	Pork salami	Frozen food	Pork	1.91 ± 0.04	1.90 ± 0.00	13.44 ± 0.37	NA
3.	P03	Smoked back bacon	Frozen food	Pork	1.96 ± 0.09	1.79 ± 0.02	14.78 ± 1.10	NA
		Negative control						
1.	N01	Streaky beef breakfast slice	Frozen food	Beef, buffalo	1.90 ± 0.07	1.62 ± 0.11	40 ± 0.00	√√
2.	N02	Chicken meatball	Frozen food	Chicken	1.92 ± 0.16	2.08 ± 0.01	39.48 ± 1.79	√√
3.	N03	Beef burger	Frozen food	Beef	1.91 ± 0.14	1.92 ± 0.10	40 ± 0.00	√√
4.	NTC	Non-template control	NA	NA	NA	NA	40 ± 0.00	NA

SD: standard deviation; C_t: cycle threshold; *40 indicates no amplification after 40 cycles; NA: not available; √: non-JAKIM halal-certified logo available on the food packages; and √√: JAKIM halal-certified logo available on the food packages.

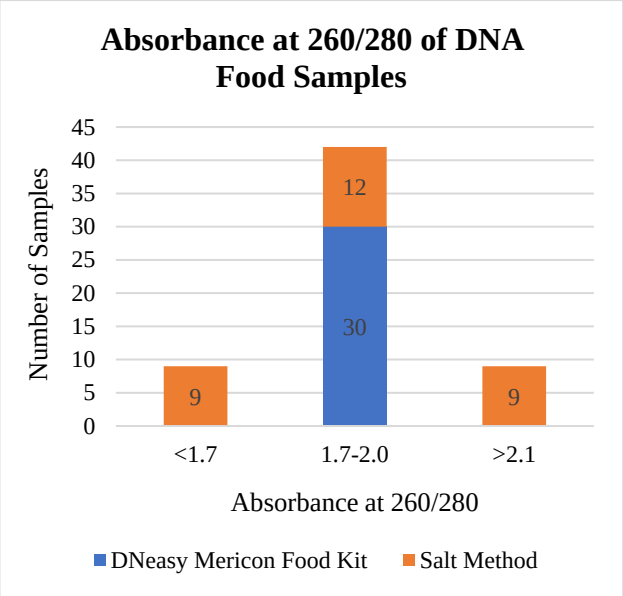

Figure 1. A_{260}/A_{280} ratios for DNA food samples extracted using both DNeasy mericon Food Kit and salt method.

nature of the food samples being associated with the seafood category, whereby seafood is known for its high protein content, comprising 15 - 20% of the edible parts of fish (Alp-Erbay and Yesilsu, 2021). The high protein content of these food samples contributed to the low absorbance ratio (below 1.7). Protein acted as inhibitor, and contamination of DNA with protein could affect qPCR (Acharya *et al.*, 2017). Therefore, the salt method used in the present work could be improved for extracting DNA from these samples by increasing the volume of proteinase K solution, which would enhance the efficiency of protein digestion, and reduce protein contamination.

Nine samples with A_{260}/A_{280} ratio of higher than 2.0, such as cabbage chicken dumpling, *pandan* chicken, taro chicken dumpling, chicken burger, beef burger, beef meatball, *pansuh* paste, *kacangma* paste, and chicken meatball, were extracted using the salt method. The A_{260}/A_{280} ratio which exceeded 2.0 typically indicated the presence of RNA contamination (O'Neill *et al.*, 2011). This was because RNA could also absorb UV light at 260 nm; hence, the high ratio could be due to presence of RNA during the purification process. As a result, this caused an increase in nucleic acid yield, which contributed to the total measurement at 260 nm, and led to an overestimation of the DNA concentration.

In comparison with the salt method, the DNeasy mericon Food Kit used a spin-column approach, in which genomic DNA was bound to the silica membrane, followed by thorough washing with washing buffer. This step removed RNAs that increased the A_{260}/A_{280} ratio while retaining the DNA bound to the silica membrane. Moreover, optimal A_{260}/A_{280} ratios were obtained by adding ribonuclease enzyme (RNase) to the elution buffer, which ensured the removal of most contaminating RNA. As per findings of Lopera-Barrero *et al.* (2008), samples subjected to RNAse treatment exhibited RNA-free DNA, in contrast to samples without RNAse treatment, whereby a substantial presence of RNA could potentially disrupt precise DNA quantitation and amplification processes. However, high RNA contamination did not affect the qPCR process. The presence of RNA interference could be attributed to the inherent instability of RNA molecules, which had a very short half-life once extracted from cells or tissues. RNA is considerably more susceptible to heat-induced degradation as compared to DNA (Tan and Yiap, 2009). Consequently, RNA degradation frequently occurs during the denaturation phase at 95°C. Additionally, it is worth to emphasise that Taq polymerase is incompatible with RNA samples, meaning that PCR is not capable of directly amplifying RNA molecules. Instead, PCR exclusively amplifies the specific targeted DNA sequences.

Determination of DNA integrity using agarose gel electrophoresis

Agarose gel electrophoresis served as a technique to confirm the presence of intact or fragmented DNA, and to verify the quality and integrity of each extracted DNA sample. Figures 2 shows the representative electrophoretic images of the DNA integrity of different food samples. These samples were subjected to DNA extraction using the DNeasy mericon Food Kit. The quality of the isolated DNA was evaluated by examining the intensity and appearance of bands on agarose gel. Generally, high-quality genomic DNA exhibits unique, well-defined bands with clear separation on the gel at their respective high molecular weights. This visual assessment aids in determining the quality and integrity of the extracted DNA. In the present work, most samples exhibited greatly expanded smears, indicating significant DNA degradation.

The degradation and fragmentation of DNA observed in the food samples may be attributed to the processing methods used, particularly the use of radiation sterilisation for meat and meat products. Various techniques such as high-pressure processing,

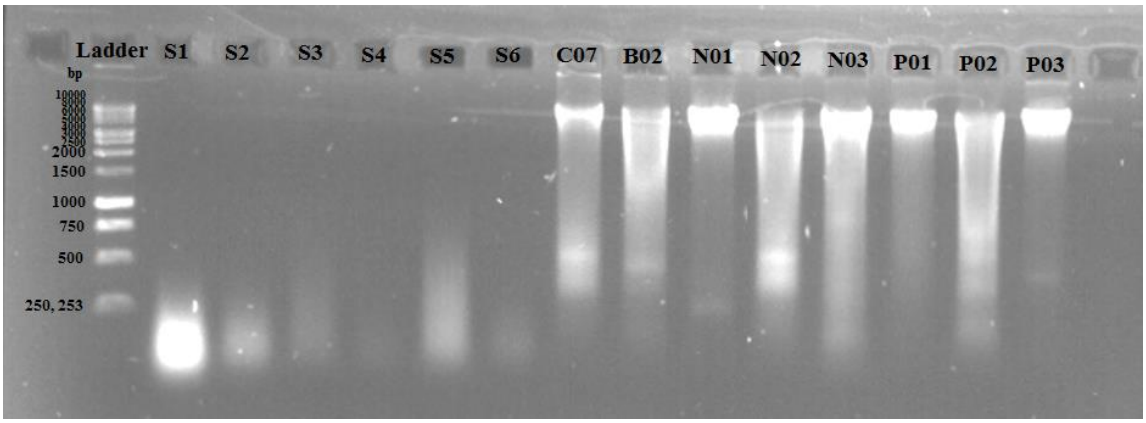

Figure 2. Representative gel electrophoretic images of DNA extracted using DNeasy mericon Food Kit. S1: spicy sardine; S2: sardine in brine; S3: fried sardine and salted black bean in soy sauce; S4: fried sardine in chilli sauce; S5: sardine in tomato sauce 1; S6: sardine in tomato sauce 2; C07: taro chicken dumpling; B02: beef meatball. Negative control with JAKIM halal-certified logo: N01: streaky beef breakfast slice; N02: chicken meatball; N03: beef burger. Positive control: P01: pork meatball; P02: pork salami; and P03: smoked back bacon.

pasteurisation, and blanching are commonly employed in the food industry to enhance microbiological safety. These food processing methods may affect the DNA constituents within food products, potentially leading to decreased DNA yield (Mohamad *et al*., 2020). Moreover, repeated cycles of freezing and thawing of food samples, which are subjected to various forms of heat, physical, or chemical treatments during production, could potentially exacerbate DNA degradation. These cumulative factors emphasise the susceptibility of DNA to degradation in processed food samples (Malentacchi *et al*., 2015).

Five samples extracted using the DNeasy mericon Food Kit showed intact bands on the agarose gel, which were taro chicken dumpling, streaky beef breakfast slice, beef burger, pork meatball, and smoked back bacon. These food products may not undergo intensive food processing techniques which could degrade DNA. Although the DNA from these food samples appeared as expanded and fragmented smears, qPCR was not affected because the amplicon size used was small (121 bp). This finding aligned with Rahman *et al*. (2015), who reported that short amplicon sizes (≤ 150 bp) offered high chances of detection.

In both DNA extraction methods employed, the findings indicated the presence of DNA in all samples, except for seasoning samples, namely *pansuh* paste and *kacangma* paste. These seasoning pastes consisted of ingredients sourced from plant-based origins. Plants exhibit far greater diversity in their chemical composition as compared to other organisms. Amongst the numerous primary and secondary metabolites found in plants, polyphenols and polysaccharides are known inhibitors which can impede the migration of DNA bands on agarose gel. Consequently, their presence can disrupt the proper running of gel electrophoresis (Särkinen *et al*., 2012). As a result, DNA might be present in these samples at lower concentrations. The absence of a band on the agarose gel did not necessarily indicate unsuccessful DNA extraction. This observation was consistent with the absorbance ratio of these samples extracted using the DNeasy mericon Food Kit, which fell within the optimal range of 1.7 - 2.0. For porcine DNA detection in the qPCR assay, only DNA extracted using the commercial kit (DNeasy mericon Food Kit) was used as a template because of the high quality of DNA obtained through this method.

Standard curve for cytb primer and probe sequences

The construction of a standard curve for the designed primer and probe sequences is crucial for qPCR assays. Typically, the optimal R^2 for a standard curve should exceed 0.99, indicating how well the data align with the standard curve. The acceptable range for the slope value in the standard curve is between -3.1 and -3.58, corresponding to an amplification efficiency ranging from 90 to 110%. The efficiency of the designed *cytb* primer and probe sequences was demonstrated by strong linear regression with a high R^2 value of 0.997. The qPCR efficiency fell within the acceptable range of 95%,

corresponding to a slope of -3.431. Consequently, the reliability of the designed *cytb* primers and probe for detecting porcine DNA at various concentrations was established (Sajali *et al*., 2022).

Detection of porcine DNA by qPCR

The porcine DNA present in various food products was identified through qPCR assay, targeting the *cytb* primer and probe sequences. Quantitative result of the qPCR assay was assessed based on the threshold cycle (C_t) values presented in Table 2. The C_t value refers to the number of cycles required for the fluorescent signal to surpass the background level, and thus indicated positive detection by the qPCR machine. The C_t levels exhibited an inverse relationship with quantity of target nucleic acids in the samples. For instance, a lower C_t value corresponds to a higher amount of targeted nucleic acid in the sample (WVDL, 2013), whereas a higher C_t value indicates a lower amount.

Only 20 out of 24 samples had a list of ingredients on the food label. The ingredients list was not available for the four samples, which were C03: sticky rice with pandan chicken; C07: taro chicken dumpling; B02: beef meatball; and F03: mackerel fishball. None of the food samples, except for the positive control samples, were labelled as containing pork species. This highlighted the importance of using molecular methods like qPCR to detect porcine DNA in unlabelled food products. In the present work, 40 cycles of complete qPCR amplification were performed. Based on the analysis, out of 24 samples, 15 samples (C01, C02, C05, C06, B01, B02, DF1, DF3, DF4, DF5, S1, S2, S3, S4, and S5) showed a mean C_t value of 40, which indicated the absence of porcine DNA, and thus could be reported as pork negative.

However, seven samples (C03, C04, S01, S03, DF2, DF6, and S6) exhibited a mean C_t value that was greater than 35 but less than 40 cycles. A previous study by Raharjo *et al*. (2019) employed a cut-off C_t value of 35 for porcine DNA detection. This threshold was chosen because a high C_t value can indicate false-positive results due to amplification or fluorescent artefact (Caraguel *et al*., 2011), or potentially suggest cross-reactivity with other species (Kesmen *et al*., 2009). This possibility was supported by Dooley *et al*. (2004), who observed cross-reactivity of chicken, beef, and lamb with pork at low percentages that ranged from 0.001 to 0.01%. In general, a C_t value of above 35 is interpreted as a weak or negative signal, suggesting the presence of either very low levels of target DNA in the sample or target DNA falling below the assay limit of detection. However, the interpretation of a C_t value as positive or negative should be based on the specific assay and its validation criteria. A C_t value of 35 is also acceptable as a cut-off value for porcine DNA detection in qPCR. Therefore, these seven samples exceeded the cut-off value of 35, and the absence of pork in the ingredient list indicated that these samples could be declared as pork negative.

Two samples, namely taro chicken dumpling (C07) and mackerel fishball (F03), had mean C_t values ± SD of less than the cut-off point of 35, which were 19.05 ± 0.72 and 28.07 ± 1.67, respectively. A low C_t value indicated that the food samples contained high concentrations of meat species-specific targets, in this case, porcine DNA. Both samples were purchased from a local restaurant. Based on Table 2, there was lack of information regarding the ingredient list and JAKIM halal-certified logo on the product label. Therefore, the presence of pork in these two samples could not be compared with the labelling statements, and thus required further validation through DNA sequencing.

The potential explanation for detecting porcine DNA in taro chicken dumpling could be unintentional cross-contamination during processing. This sample was bought from a local restaurant in Sibu which sold various kinds of *dim sum*, including shrimp *shumai*, pork dumpling, and chicken *shumai*. Consequently, improper handwashing and the use of shared equipment, such as knives, meat grinders, and cutting boards when processing chicken and pork simultaneously during *dim sum* preparation could lead to the cross-contamination of pork with taro chicken dumpling. Furthermore, fishballs and meatballs made with minced meat are commonly targeted for adulteration, as reported by Orbayinah *et al*. (2020). Meatballs can be intentionally or unintentionally adulterated with pork, leading to contamination with porcine DNA. The absence of product labelling information regarding the presence of pork poses potential risks to Muslim customers and individuals with dietary restrictions or pork allergies.

In the present work, both positive and negative controls were incorporated to ensure validity of the results, which were deemed satisfactory. All positive controls containing pork meat and its derivatives, as declared in the ingredients, were positive for porcine DNA, with mean C_t value ± SD that range from 13.44

± 0.37 to 14.78 ± 1.10. Additionally, the negative control labelled with the JAKIM halal-certified logo with no pork shown in the food labelling had mean C_t ± SD value that ranged from 39.48 ± 1.79 to 40 ± 0.00, which indicated the absence of porcine DNA detected in the food samples (Rahman, 2022). The analysis confirmed that all 25 samples, including the negative control with the JAKIM halal-certified logo, were tested negative for porcine DNA. Figure 3 illustrates the presence of the halal logo on product labels, and its association with the presence or absence of porcine DNA in food samples.

The remaining five samples (fried dace with salted black bean 1, fried dace with salted black bean 3, fried dace with salted black bean 6, sardine in tomato sauce 1, and sardine in tomato sauce 2) were featured with the halal logo on their labels, but they were not JAKIM-certified. Referring to the product's country of origin, these samples were imported from China. Even the JAKIM-certified halal logo was absent on these five samples; the halal logo printed on the product labelling was the foreign halal logo certified by the Shandong Halal Certification Service (SHC), except for fried dace with salted black bean 1. The use of this halal logo has been approved by JAKIM, which enables Muslim consumers to be self-assured that the products they consume are permissible. Moreover, based on qPCR analysis, the absence of porcine DNA in these five samples verified that these samples can be declared as halal.

In addition, the 17 remaining samples which lacked the JAKIM halal-certified logo also demonstrated the absence of porcine DNA based on the qPCR results. Despite this, the absence of the JAKIM halal-certified logo raises doubts amongst Muslim consumers regarding the halal status of these products. This is because the concept of halal encompasses more than just the absence of pork; it also includes other factors, such as ensuring the animals are healthy and slaughtered following the Malaysian Standard on Halal Food (MS1500:2009). Food manufacturers should comply with and adhere to the halal regulations set forth by JAKIM, and consistently ensure the halal status of their products (Rahayu and Abinawanto, 2022).

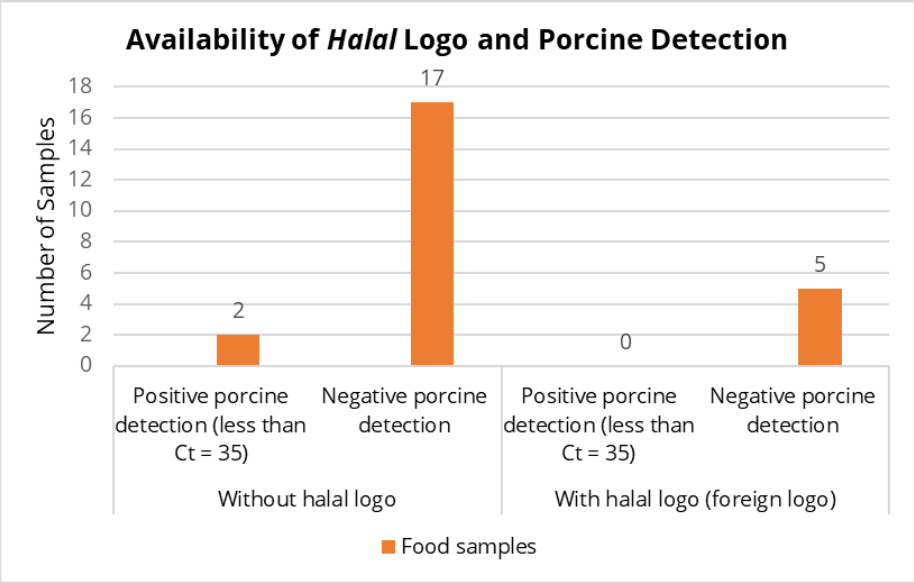

Figure 3. Porcine DNA detection and its relationship with availability of halal logo on product packaging.

Validation of positive samples

Two samples (C07 and F03) that were tested positive for porcine DNA in the qPCR assay were prepared for DNA sequencing. Unpurified PCR products were sent to Apical Scientific Sdn. Bhd., Selangor, Malaysia, for DNA sequencing. The resulted chromatogram was examined using FinchTV software, and the sequenced results were analysed through BLAST in the NCBI database. The query sequences of the samples C07 and F03 indicated 95.29 - 100% and 94.74 - 96.70% percentage identities, respectively, with the target sequence in the NCBI database, thus indicating *Sus scrofa domesticus* as the species. Positive control also revealed a high percentage identity of 85.11 - 98.80% with *Sus scrofa domesticus* in the NCBI database, which validated this finding. Therefore, it could be verified that samples C07 and F03 contained *Sus scrofa domesticus* and its derivatives.

Conclusion

The consumption of food products adulterated with pork is prohibited in Islam, and could potentially trigger allergic reactions in specific individuals. Mislabelling of food products could lead to the incidental consumption of pork by consumers. In the present work, the presence of porcine DNA in selected commercial food products was determined using a qPCR assay that utilised a custom-designed TaqMan probe targeting the *cytb* sequences. DNA extraction from food samples was conducted using both the DNeasy mericon Food Kit and salt method, with a subsequent comparison of their outcomes in terms of DNA purity and integrity. Results indicated that the DNeasy mericon Food Kit was the preferred choice for DNA extraction, as it consistently yielded DNA samples with optimal purity, quality, and suitability for downstream analyses. Due to variations in the food matrices, the DNA extraction of certain food products, such as seafood and seasonings, was challenging. For example, the seasoning contained a plant-based inhibitor, which required modification of the DNA extraction protocol. Therefore, future work is required to determine suitable extraction methods for these samples to ensure the isolation of pure DNA for downstream applications.

The qPCR assay demonstrated that porcine DNA was detected in two samples: C07, taro chicken dumplings (no halal logo and ingredient list); and F03, mackerel fishball (no halal logo and ingredient list). The validation results of these two samples indicated a 94.74 - 100% similarity of the BLAST query sequence with the NCBI database sequence for *Sus scrofa domesticus*. Subsequent investigations should involve quantifying the concentration of porcine DNA in positive food samples to distinguish between accidental and intentional adulteration. The present work provided initial insights into the halal status of specific food products, and demonstrated the importance of a surveillance programme by regulatory authorities in ensuring the authenticity of meat and its traceability back to its origin of production.

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