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Research Article

Evaluation of Species-Specific PCR Primers for Detecting Pork DNA in Food Seasoning Products

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ABSTRACT

Food adulteration remains a critical issue, particularly in highly processed foods where DNA degradation reduces the effectiveness of species identification. This study evaluated the performance of three different lengths of species-specific primers (398, 288 & 149 bp) targeting the mitochondrial cytochrome B gene in pork and aimed to determine the most effective primer for detecting pork DNA in food seasoning products using species-specific Polymerase Chain Reaction (PCR). Furthermore, species-specific primers for bovine and chicken were applied as controls to confirm species identification. The DNA was extracted from raw meat, binary mixtures, and seasonings. Sensitivity was tested with low pork concentrations, and the applicability of pork-specific primers was further evaluated in seasoning products without halal certification to assess potential pork adulteration. The results show that the DNA extracted from raw meat samples exhibited high purity (OD260/280:1.82 –2.00) and concentration (114.83 to 257.16 ng/µL), whereas food seasoning products yielded extremely lower values of purity (OD260/280:0.97 – 1.81) and concentration (2.75 to 66.18 ng/µL). Despite DNA degradation in processed foods, PCR products remain detectable. The shortest 149 bp primer demonstrated the highest sensitivity among the primers, successfully detecting pork DNA at a minimum concentration of 1% (w/w) in a binary meat mixture. The results demonstrated the absence of pork DNA in all non-pork labelled samples. However, the detection of chicken DNA in fermented pork cube samples indicated potential mislabelling or cross-contamination during processing. These findings emphasise the importance of primer selection for detecting highly degraded DNA. This also underscores the applicability of species-specific PCR as a practical and robust approach for routine food authentication, providing a valuable tool to ensure halal compliance within the food industry.

Key words: Amplicon length, cytochrome B gene, food adulteration, pork DNA, species-specific PCR

INTRODUCTION

Recent advancements in food science and technology have expanded the variety and applications of food products, including seasonings. Food seasonings, such as spices, bouillon cubes, and mixed herbs, have been widely consumed because of their flavour-enhancing properties, which improve the aroma and taste of food (Śmiechowska *et al*., 2021). Spices have also been used as preservatives in the food industry (Jessica Elizabeth *et al*., 2015). The addition of preservatives is primarily intended to extend the shelf life of food products (Momtaz *et al*., 2023) by inhibiting bacterial growth and preventing spoilage.

However, rapid advancements in food processing have made it challenging to trace the origin of animal ingredients, especially pork, due to complex processing methods and ingredient mixing (Muflihah *et al*., 2023). Processed food products tend to be altered by physical, chemical, and biochemical factors during manufacturing (Deb-Choudhury *et al*., 2020; Igual & Martínez-Monzó, 2022). Most processed meat products are manufactured from comminuted meat, making it difficult to identify animal species (Sreenivasan Tantuan & Viljoen, 2021). This lack of traceability increases the risk of food fraud, including the mislabelling and use of undeclared animal ingredients.

Food fraud, including ingredient mislabelling and the use of counterfeit halal logos on food packaging, is a growing global concern. Mislabelling can mislead consumers into making unintended choices and may even lead to serious religious implications in Islamic countries (Fuseini *et al*., 2017). Many religious dietary laws strictly regulate food consumption, with Islamic and Jewish laws prohibiting pork and Hinduism prohibiting beef (Sajali *et al*., 2018). Additionally, from a health perspective, unintentional consumption of pork can be harmful to individuals with pork allergies, a condition known as pork-cat syndrome. (Turner *et al*., 2013). This underscores the need for enhanced food authentication protocols to ensure compliance with dietary restrictions and public health.

Considering these issues, reliable techniques for detecting pork derivatives in complex food matrices are crucial. Recently,

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the main approaches used to identify animal-derived components are protein-based and DNA-based methods (Yin *et al*., 2020). While protein-based techniques have been employed for meat authentication, this method, including chromatographic methods (Dewi *et al*., 2023) and Fourier Transform Infrared (FTIR) spectroscopy (Nurani *et al*., 2022), is limited by low sensitivity and detection challenges due to protein denaturation during high-temperature processing (Yin *et al*., 2020; Al-Shaibany *et al*., 2022). In contrast, deoxyribonucleic acid (DNA)-based methods are preferred over protein-based methods because of their higher stability (Wu *et al*., 2020) and are more resilient to heat and processing conditions (Članjak–Kudra *et al*., 2021). Although several studies have focused on detecting pork DNA in various food products, data regarding pork DNA detection in food seasoning products remain scarce. This is due to highly degraded DNA and the inability to extract sufficient DNA that can be amplified by PCR (Sreenivasan Tantuan & Viljoen, 2021).

Among DNA-based detection methods, species-specific PCR is preferred due to its advantages. Compared to techniques such as Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR), species-specific PCR offers cost-effectiveness, high specificity, and rapid results. RAPD PCR requires strict and consistent PCR amplification conditions, and its reproducibility is often challenging (Amiteye, 2021). Additionally, PCR-RFLP involves a complex procedure (Gargouri *et al*., 2021), making it labour-intensive, time-consuming, and less practical than species-specific PCR techniques. Real-time PCR, which is highly specific and sensitive, is expensive regarding instrumentation and reagents, limiting its accessibility, particularly in resource-limited settings (Mote *et al*., 2021).

The detection of pork DNA in complex food matrices underscores the importance of optimising the PCR, with primer selection being crucial for both efficiency and specificity. The choice of primers, particularly their length and target region, significantly affected the success of DNA amplification. Studies have shown that short amplicons targeting the *cytb* gene in pork are highly effective for species detection even in cases where DNA is highly fragmented. Orbayinah *et al*. (2019) demonstrated that short amplicons, less than 250 bp, improved the amplification efficiency of PCR. Similarly, Uddin *et al*. (2021) reported that primers with smaller amplicon sizes resist thermal treatments, including autoclaving, due to the greater thermodynamic stability of short amplicon sequences than longer amplicons (Hossain *et al*., 2016). The *cytb* is especially valuable for species identification because of its conserved nature and species-specific mutation sites, which enable precise differentiation between domestic and wild animals (Farag *et al*., 2020).

Therefore, this study aimed to evaluate the efficiency of three species-specific primers targeting the *cytb* gene of pork, with amplicon lengths of 149, 288, and 398 bp, using PCR. Additionally, it aimed to assess the potential for pork contamination in food seasoning products.

MATERIALS AND METHODS

Sample collection

This study was conducted at the Laboratory of Molecular Microbiology, Faculty of Resources Sciences and Technology, University of Malaysia Sarawak. Three raw meat samples from three target animal species, namely pork (*Sus crofa domesticus*), chicken (*Gallus gallus domesticus*), and bovine (*Bos taurus*), were purchased from wet markets in Kota Samarahan, Sarawak, as reference samples. A total of eight samples of food seasoning without the Halal certification logo were used in this study. These samples were then transported to the laboratory and kept at −20°C before analysis.

Pork-specific primer sensitivity assessment preparation

The performance of the species-specific PCR assay and the sensitivity of pork-specific primers were evaluated using a binary mixture of pork with bovine meat, where pork was deliberately adulterated following a study by Suadi *et al*. (2020) with minimal modification. The percentage of pork: bovine ratios is as follows: (i) 1:99, (ii) 5:95, and (iii) 10:90 pork: bovine ratios (w/w) with a total weight of 500 mg. Similar ratios were also applied to chicken samples (pork: chicken). Low percentages (%) were chosen to mimic realistic scenarios of low-level food adulteration, emphasising the importance of detecting even trace amounts of undeclared pork. Only three pork-specific primers were assessed, while chicken and bovine primers were excluded from the analysis.

Pork adulteration detection assessment

Eight food seasoning products without a halal logo were used in this study. All materials used during sample preparation were disinfected with 70% ethanol to minimise contamination. The samples were minced using a flame-sterilised knife. Approximately 500 mg of the sample was weighed using an analytical balance (Joan Lab, China) and transferred into 2.0 mL microcentrifuge tubes for subsequent analysis.

DNA extraction and quantitation

The DNA from food seasoning products was extracted, adhering to the instructions provided by the manufacturer of the DNeasy Mericon Food Kit (Qiagen, Germany) with minimal modification. The concentration and purity of the extracted DNA were quantified using a DS-11 Series Spectrophotometer (DeNovix, USA) by measuring absorbance at 260 nm (A260) and 280 nm (A280). A_{260}/A_{280} ratio values between 1.8 and 2.0 indicate pure DNA, free from contaminants (Kusnadi *et al*., 2024). Additionally, the extracted DNA was required to yield at least 20 ng, as suggested by Sophian *et al*. (2021), who reported that DNA yields above this threshold indicate efficient extraction.

Species-specific PCR amplification

Three pairs of species-specific oligonucleotide primers targeting the *cytb* gene were utilised to detect pork DNA. Additionally, two primer sets specific to bovine and chicken species were used as a control for species identification. The information on these primers is detailed in Table 1.

Table 1. The species-specific primers used in this study

Primer Name	Oligonucleotide Sequence (5' to 3')	DNA bases (bp)	Amplicon Size (bp)	References
Pork 1	GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA	38	398	Matsunaga *et al*., 1999
	GCT GAT AGT AGA TTT GTG ATG ACC GTA	27		
Pork 2	ATCCGACACAACAACAGCATTCTCCT	26	288	Oanh *et al*., 2017
	GCTGATAGTAGATTTGTGATGACCGTA	27		
Pork 3	ATG AAA CAT TGG AGT AGT CCT ACT ATT TAC C	31	149	Dooley *et al*., 2004
	CTA CGAGGT CTGTTC CGA TAT AAGG	25		
Bovine	CGGCACAAATTTAGTCGAAT	20	120	Hossain *et al*., 2016
	TGGACTATGGCAATTGCTATG	21		
Chicken	AGC AAT TCC CTA CAT TGG ACA CA	23	133	Dooley *et al*., 2004
	GAT GAT AGT AAT ACC TGC GAT TGC A	25		

The *cytb* gene was amplified using GoTaq® Green Master Mix (Promega, USA) in a reaction volume of 25 µL. The reaction contained 12.5 µL of master mix, 8.5 µL of nuclease-free water, 2 µL of DNA template, and 1 µL each of forward and reverse primers specific to the *cytb* gene. The species-specific PCR was conducted on a T100 Thermal Cycler (BioRad, USA) with the following conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 45 sec, and extension at 72°C for 1 min, concluding with a final extension at 72°C for 5 min.

Gel electrophoresis

Five µL of PCR products were electrophoretically determined using a 2% agarose gel in 1X TBE buffer and stained with SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, USA). Gel electrophoresis was done for one hr at a constant voltage of 80 V. A ready-to-use 100 bp DNA (ExactMark, Singapore) was utilised as a molecular marker, and the images obtained were then visualised using a UV transilluminator (Cleaver Scientific, UK).

RESULTS AND DISCUSSION

Quantitation of extracted DNA

The purity and concentration of the extracted DNA are listed in Table 2. The results revealed that the DNA concentration of raw DNA samples ranged from 114.83 to 257.16 ng/µL, and the purity (OD260/280 values) of raw DNA samples ranged from 1.82 to 2.00. In contrast, DNA extracted from food seasoning products exhibited lower purity values, ranging from 0.97 to 1.81, and concentrations between 2.75 and 66.18 ng/µL.

Table 2. Concentration and purity of the extracted DNA

Categories	Samples	Concentration of DNA (ng/µL)	Purity Index (A_{260}/A_{280})
Raw meat	Pork meat	114.83	1.99
	Bovine meat	148.39	1.88
	Chicken meat	257.16	2.00
Deliberate adulteration of pork meat with other meat	1% raw pork in bovine	157.21	1.87
	5% raw pork in bovine	154.20	1.86
	10% raw pork in bovine	143.74	1.82
	1% raw pork in chicken	346.36	1.99
	5% raw pork in chicken	372.52	2.00
	10% raw pork in chicken	208.87	1.94
Food seasoning	Pork cube	2.75	0.97
	Bovine cube	3.55	1.25
	Chicken cube	6.42	1.32
	Fermented pork powder	12.28	1.60
	Chicken powder	25.30	1.74
	Black pepper powder	6.98	1.73
	Garlic salt	66.18	1.72
	Turmeric powder	16.80	1.81

The extremely low values in processed food compared to those in fresh meat samples may be due to DNA degradation caused by food processing practices, including extensive thermal processing (Sajali *et al*., 2022) during sterilisation. Prolonged heat exposure and elevated temperatures progressively reduce the size of DNA fragments via thermal degradation (Şakalar *et al*., 2012). Despite the low DNA concentration and purity, the experiment proceeded with PCR amplification. This is because the DNA concentration and absorbance ratio do not necessarily correlate with PCR amplification (Piskata *et al*., 2019). Thus, even degraded DNA could serve as a viable template for amplification under optimised conditions.

Pork-specific primer sensitivity assessment

A sensitivity assessment was implemented by simulating real-world food adulteration scenarios by preparing different proportions of a binary mixture of pork and other meat species. Sensitivity assessment was implemented by simulating real-world food adulteration scenarios by preparing different proportions of a binary mixture of pork and other meat species. As shown in Figures 1(a) and (b), all primer sets successfully amplified pork DNA from the tested mixtures, producing clear bands at the expected amplicon sizes of 398 bp, 288 bp, and 149 bp. This indicated the presence of pork in both the pork-chicken and pork-beef mixtures. These findings further confirmed that pork adulteration was detectable at concentrations as low as 1% in both mixtures, with no observable reduction in the band intensity. Additionally, the results highlight the effectiveness of species-specific PCR with primers for the detection of trace amounts of pork contamination.

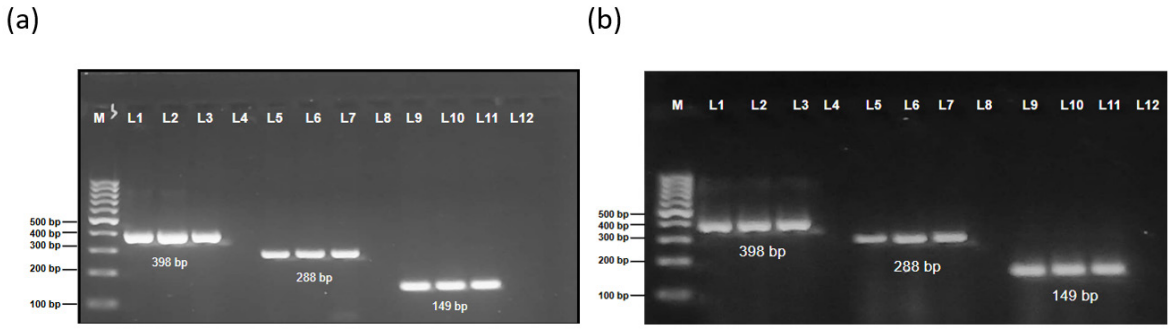

Fig. 1. Gel electrophoresis results for pork DNA detection in adulterated meat mixtures. (a) Pork-chicken mixture, (b) Pork-bovine mixture. M: Marker; Lane 1-3: Pork 1 primer (398 bp); L1: 1%, L2: 5 %, L3: 10% pork adulteration; Lane 5-7: Pork 2 primer (288 bp); L5: 1%, L6: 5%, L7: 10% pork adulteration; Lane 9-11: Pork 3 primer (149 bp); L9: 1%, L10: 5%, L11: 10% pork adulteration; Lanes 4, 8, and 12 were negative control.

These observations are consistent with the findings of Suadi *et al*. (2020), who reported that pork in other meat mixtures could be identified in up to 1% (w/w) adulteration. In addition, Ha *et al*. (2017) documented that pork can be traced at 1%, even after heat treatment. Cahyadi *et al*. (2021) also reported that contamination in pork–bovine meat mixtures could be detected at adulteration levels as low as 1% (w/w) in raw and cooked samples. Other researchers have used multiplex PCR (Yang *et al*., 2022) and real-time PCR (Kim *et al*., 2023) to detect adulteration in meat mixtures using a DNA dilution series. However, a deliberate binary mixture of the two meat species was preferred over a series of dilutions of DNA for the sensitivity assessment because this approach was more in line with the real circumstances of meat adulteration in the marketplace (Wang *et al*., 2019).

Notably, the Pork 2 primer generated a moderately intense band at 288 bp (Figures 1 (a) and (b)). The Pork 1 and Pork 3 primers produced bands of similar intensity, appearing thick and indicating strong amplification at 398 and 149 bp, respectively. However, an additional faint band at 398 bp was observed with the Pork 1 primer, despite the similar DNA concentrations and purities across all primers. This suggested non-specific amplification. Among the three primer sets, the Pork 3 primer (149 bp) showed the strongest amplification, without any additional non-specific products. This evidence indicates that this primer was the most sensitive for detecting pork DNA at lower concentrations than the Pork 1 and 2 primers.

Pork adulteration detection assessment in food seasoning products

The analysis employed species-specific primers targeting the mitochondrial *cytb* gene of pork, namely Pork 1 (398 bp), Pork 2 (288 bp), and Pork 3 (149 bp), as listed in Table 1. These published primers were assessed for their ability to detect pork DNA in the food seasoning products. In addition, bovine (120 bp) and chicken (133 bp) primers were included as control markers to confirm the presence of the declared animal species, as indicated by product labelling. This will help trace any potential mislabelling and contamination. The amplicon sizes of the DNA bands corresponding to the target genes were compared with those of the positive control. Reference samples of pork, bovine, and chicken meat were utilised as positive controls to ensure the reliability of the results, whereas nuclease-free water was used as a negative control to detect any potential contamination during the experimental procedure.

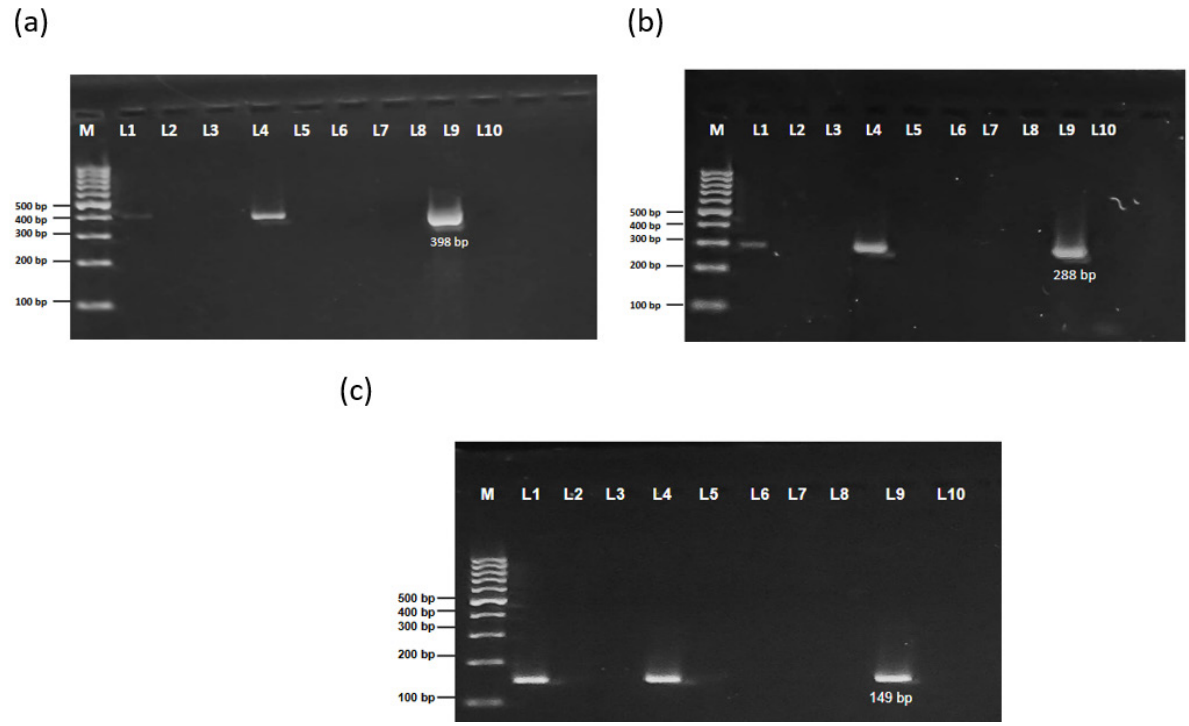

Fig. 2. Gel electrophoresis results for species-specific PCR detection in food seasoning products. Species-specific PCR amplification targeting pork DNA (a) Pork 1, 398 bp, (b) Pork 2, 288 bp, (c) Pork 3, 149 bp. M: DNA marker; L1: pork cube; L2: bovine cube; L3: chicken cube; L4: fermented pork powder; L5: chicken powder; L6: black pepper powder; L7: garlic salt; L8: turmeric powder; L9: positive control; (a) pork meat, (b) bovine meat, (c) chicken meat; L10: negative control.

The results of the detection of pork species from these samples are presented in Figures 2 (a), (b), and (c). The Pork 3 primer successfully amplified DNA from pork cubes and fermented pork powder samples. No pork DNA was detected in the other tested food seasoning products, as indicated by the absence of DNA bands on the agarose gel. These findings suggest that there was no intentional substitution or contamination involving pork-derived components in these samples, even though these products were not labelled with a halal certification logo on their packaging. This outcome further supported the sensitivity and reliability of the 149 bp amplicon in detecting pork DNA in highly processed products.

However, differences in band intensity were observed between the primer sets. Pork 1 and Pork 2 primers, amplifying 398 bp and 288 bp amplicons, respectively, generated faint bands in pork cube samples, as shown in Figures 2 (a) and (b). In contrast, the Pork 3 primer, which targeted a shorter 149 bp region, generated a clear and high-intensity band, as illustrated in Figure 2 (c). This observation aligns with a previous finding indicating that species identification can be efficiently conducted using short fragments of mitochondrial *cytb*, particularly in degraded or minute DNA samples (Andrejevic *et al*., 2019). Amaral *et al*. (2014) also reported that primers amplifying shorter DNA fragments can exhibit significantly higher sensitivity, even when working with highly fragmented DNA samples.

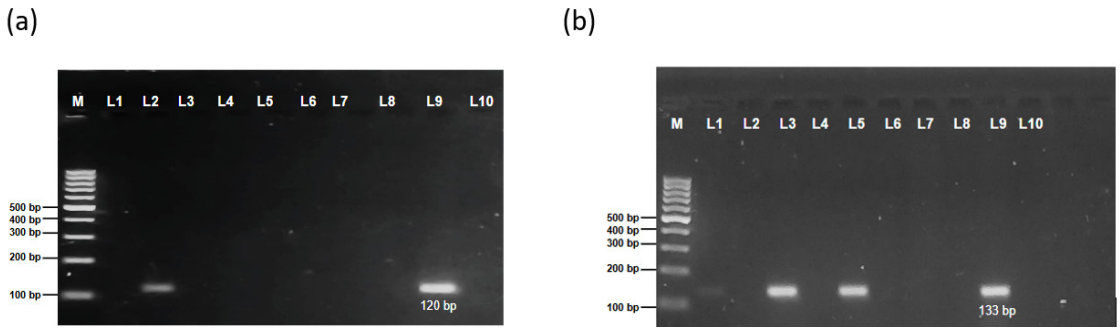

Fig. 3. Gel electrophoresis results for species-specific PCR detection in food seasoning products. (a) species-specific PCR amplification targeting bovine DNA (120 bp). (b) species-specific PCR amplification targeting chicken DNA (133 bp). M: DNA marker; L1: pork cube; L2: bovine cube; L3: chicken cube; L4: fermented pork powder; L5: chicken powder; L6: black pepper powder; L7: garlic salt; L8: turmeric powder; L9: positive control; (a) pork meat, (b) bovine meat, (c) chicken meat; L10: negative control.

As shown in Figure 3 (a), the presence of bovine DNA in food seasoning was depicted by the DNA band at 120 bp targeting the *cytb* gene on the agarose gel image. Only bovine-derived samples, including bovine cubes, were observed. This observation indicates the presence of bovine DNA in this sample. No band was detected in the other tested food seasoning samples or the negative control, indicating the absence of bovine DNA in these samples.

In contrast, Figure 3 (b) shows that the chicken primer detected bands at 133 bp, not only in chicken-derived samples, such as chicken cubes and chicken powder, but also in pork cubes. Although the band detected for the pork cube was faint, this observation suggests the presence of chicken DNA. No band was observed in the bovine cubes, other plant-based seasonings, or the negative control. Additionally, the inclusion of appropriate negative controls indicated that no contamination had occurred throughout the experimental process.

These results are summarised in Table 3, demonstrating that the detection of chicken DNA in pork cubes may be attributed to cross-contamination or unintentional adulteration. This may be due to the simultaneous processing of chicken, bovine, and pork in the same factory using shared equipment and improper handling practices (Sajali *et al*., 2022).

Table 3. Detection of pork adulteration in food seasoning products by gel electrophoresis

Sample Information		Species-specific PCR results				
Product Description on Label	Main Ingredients	Pork 1 (398 bp)	Pork 2 (288 bp)	Pork 3 (149 bp)	Bovine (120 bp)	Chicken (133 bp)
Pork cubes	Pork	+	+	+	-	+
Bovine cubes	Bovine	-	-	-	+	-
Chicken cubes	Chicken	-	-	-	-	+
Fermented pork powder	Pork	+	+	+	-	-
Chicken seasoning powder	Chicken	-	-	-	-	+
Black Pepper	Black Pepper	-	-	-	-	-
Garlic Salt	Garlic	-	-	-	-	-
Turmeric Powder	Turmeric	-	-	-	-	-

These findings emphasise the critical role of accurate food labelling in guiding consumer decisions and ensuring the authenticity and traceability of food products (Basanisi *et al*., 2020). Proper labelling provides essential information regarding ingredients, nutritional content, and includes important details such as batch numbers, shelf life, and storage conditions to ensure food safety (Moreira *et al*., 2021). As food labelling significantly influences consumer preferences (Bandara *et al*., 2016), it is crucial for consumers to carefully review the information to ensure that products are aligned with their dietary needs, such as vegetarian diets or specific health conditions, such as diabetes *(*Tonkin *et al*., 2016*)*.

While this study noted the lack of a halal logo on packaging, it was compulsory in many countries to declare specific meat species on labels to address concerns regarding food allergies, religious dietary requirements, and food fraud prevention (Soares *et al*., 2013). Product authenticity is not necessarily guaranteed by labelling or certification (Sreenivasan Tantuan & Viljoen, 2021). Some irresponsible meat vendors have replaced high-value meat with lower-cost (Doosti *et al*., 2014) or undesirable species to gain economic profit. Therefore, it is essential to develop an accurate method for detecting pork contamination to prevent food adulteration.

Although proper labelling plays a crucial role in consumer protection, it must be supported by reliable detection methods to verify product authenticity and prevent mislabelling. In this study, despite the potential for DNA degradation caused by high-temperature processing, pork, chicken, and beef DNA in food seasoning remained amplifiable. This was evidenced by band visualisation at the expected amplicon sizes of 398, 288, 149, 120, and 133 bp for pork, bovine, and chicken, respectively. This detection is possible because species identification can be efficiently conducted using short fragments of the mitochondrial *cytb* gene, particularly in minute or degraded DNA samples (Andrejevic *et al*., 2019). Furthermore, shorter amplicon sizes can amplify DNA even after extensive thermal processing, whereas longer amplicon sizes are more susceptible to degradation (Rashid *et al*., 2015). While Dooley *et al*. (2004) validated the 149 bp primer in raw meat samples, our study extended its application to complex food seasoning matrices, where it consistently detected pork DNA despite DNA degradation. This highlights its broader applicability in diverse food products.

The development of species-specific PCR targeting the *cytb* gene has enabled the precise identification and differentiation of animal species in various applications. This advancement was due to the increased reliability of the mitochondrial *cytb* gene, owing to its abundance in cells (Farag *et al*., 2020). These findings showed that species-specific primers with shorter amplicon lengths were highly effective for detecting fragmented DNA in complex matrices. These results demonstrate that degraded DNA can be effectively amplified when appropriate primers and optimised extraction methods are used, highlighting the importance of method selection in DNA analysis.

CONCLUSION

This study demonstrated the effectiveness of short species-specific primers targeting the *cytb* gene of pork (398 bp, 288 bp & 149 bp) for detecting pork DNA in food seasoning products, even though the DNA was highly degraded. The species-specific PCR assay used in this study offers a practical, cost-effective alternative to more advanced techniques, such as real-time PCR, ensuring accessibility for routine screening in food authenticity and halal compliance monitoring. Further studies could expand this work by detecting other non-halal substances in other food types.

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ETHICAL STATEMENT
Not applicable.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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