DUPLEX DETECTION OF AVIAN AND CHICKEN USING COMMON PRIMER-PCR IN PROCESSED MEAT PRODUCTS

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Abstract

Meat fraudulent practice has been proven to damage the quality of the food and consumer's health. Being widely available at a lower cost, chicken meat has been frequently used as a meat substitute in meat-based food products. As a solution, a duplex polymerase chain reaction (PCR) assay employing a common primer coupled with a unique adapter sequence was developed for simultaneous chicken-specific and avian universal detection. The assay targeting 322 and 209 bp of melanocortin 1 receptor (MC1R) gene sequence was optimized with high specificity against a range of avian and meat species and a high limit of detection (LOD) of 0.01 ng. Across 42 commercial meat products tested, chicken meat was detected in seven non-avian samples, where only two of them declared the presence of chicken meat on the label. The practical duplex PCR assay can be potentially applied for routine practice in qualitative investigation of contamination or adulteration in processed meat products.

Keyword: Avian, chicken, duplex PCR, MC1R gene, meat

Introduction

The high demand and competitive market in meat-based food products may prompt food manufacturers to optimize their production economically, which leads to fraudulent practices by substituting meat with different types of low-cost meats or meat analogs. Fraudulent awareness has arisen along with the demand for high-quality food, particularly meat and meat products. Incorrect labeling may also have implications on health, where certain types of meat, for example, chicken meat, have been reported to be an allergen to certain people (Wanniang et al., 2022; Klug et al., 2020). Thus, the validation of meat origin in food needs to be conducted and controlled by legal authorities by means of robust, accurate, and sensitive methodologies capable of assuring that fraudulent or accidental mislabeling does not arise. Following this issue, several international bodies, including the World Health Organization (WHO) and World Trade Organization (WTO), encourage the Association of South East Asian Nations (ASEAN) countries to harmonize their food labeling regulations with international standards, guidelines and recommendations, such as those for Codex Alimentarius (Kasapila & Shaarani, 2011).

In order to identify meat origin, many studies have developed molecular methods based

on protein or DNA analyses. Although the heat-stable protein detection method is now available, DNA-based methods have become more important and are less affected by the type of industrial processing implemented. Polymerase chain reaction (PCR) technique applications, which are specific, sensitive, and applicable to many types of food products, including heat-processed products, have been extensively investigated. Commonly, PCR coupled with restriction-fragment length polymorphism (RFLP) and sequence analysis have been widely used for speciation testing of meat products (Haider et al., 2012; Doosti et al., 2014; Gargouri et al., 2021). However, they are time-consuming and costly for routine species identification in mixed samples. On the contrary, multiplex PCR can detect multiple species in a single reaction step (Matsunaga et al., 1999; Kitpipit et al., 2014; Izadpanah et al., 2018), rendering it a faster, cheaper, and easier alternative to PCR-RFLP (Haider et al., 2012; Doosti et al., 2014; Gargouri et al., 2021) and sequence analysis (Galal-Khallaf, 2021).

Even though the multiplex PCR system has become a distinguished approach in the identification of meat species in food and animal feed, it faces several limitations, including complexity in the reaction, low or variable amplification efficiency due to self-inhibition among different primers, non-specific and partial amplification, and low universality (Bai et al., 2009). Therefore, several multiplex strategies have emerged to enhance the diagnostic capacity and solve the weaknesses of general multiplex PCR, such as common single primer multiplex PCR (Xu et al., 2008), common primer multiplex PCR (Bai et al. 2009), universal multiplex PCR (Wen & Zhang, 2012), universal primer multiplex PCR (Xu et al., 2012), and single universal primer multiplex ligation-dependent probe amplification (Shang et al., 2013). Most of these methods utilize the application of a common primer and/or common adapter to compensate for the limitations and avoid the decrease in amplification efficiency of the multiplex PCR reaction system. This approach, which is commonly used in microbiology (Tao et al., 2020) is still scarcely utilized in food authentication.

Considering chicken as a relatively cheap and widely consumed meat substitute, this study developed a common primer duplex PCR assay for the co-detection of chicken and avian groups. Comprising a pair of chicken-specific primers and an avian universal primer targeting the melanocortin 1 receptor (MC1R) gene, the assay serves as a model for qualitative meat identification in moderately processed meat products.

Materials and Methods

Sample collection

Fresh raw meats (chicken, duck, turkey, cattle, deer, goat, pig, and rabbit) and 42 commercial processed meat products were obtained from local retail markets. Samples were cut into small pieces (approximately 1 mm^2) with sterile surgical blades and stored at -20 °C until use.

DNA preparation

The meat samples were homogenized in separate tubes. The DNA of raw meats was extracted with the QIAamp DNA Mini kit (Qiagen, Germany) method following the manufacturer's protocol. Meanwhile, the DNA extractions of the meat products were conducted according to Wu et al. (1995) rapid method of extracting DNA from animal tissues. The DNA concentration was determined using fluorescence dye (Quant-iTTM PicoGreen® dsDNA Assay Kit, Life TechnologiesTM) and measured using a microplate reader (Infinite® M200, NanoQuant, Tecan) according to the manufacturer's protocol. DNA purity was estimated by measuring the A260/A280 and A260/A230 ratios using a spectrophotometer (Eppendorf® BioPhotometer)

with an Eppendorf μCuvette™ G1.0 for a microvolume measurement.

Primer design

Species-specific primers were designed using the MC1R gene as the target. The gene sequences were obtained from the GenBank database (www.ncbi.nlm.nih.gov) and analyzed through sequence alignment using Bioedit version 7.1 to find the suitable target site. Sequence for the forward primer, MCF2, was selected to be shared by chicken and all included members of the avian group: chicken (AY235570.1), turkey (GU90506 3), duck (HQ699486), goose (AY521209). In contrast, the reverse primers for avian (MAviR) and chicken (MChiR) were designed separately. The specific primers (MAviR and MChiR) were differentiated at the 3′-end to ensure successful specific amplification of the avian group. The amplification of the chicken gene resulted in two DNA fragments (chicken and avian), rendering the semiqualitative approach more reliable. These selected primer sequences were analyzed in silico for specificity, secondary structures, possible priming sites, and optimal annealing temperature using Oligo Analyzer 3.1 (https://sg.idtdna.com/analyzer/Applications/OligoAnalyzer/). Details of the primers used in this study are given in **Table 1**. Chicken-specific adapter primer (MChiRa) and avian universal adapter primer (MAviRa) were linked to an 18-bp common adapter CaR sequence (5′-CCTTCCTTCCTTCCTTCC-3′) at the 5′-end. A common forward primer (MCF2) was purposely designed longer to increase the specific binding to the target MC1R sequence of all animals in this study, and the ratio of base mismatches within the region will bring the annealing temperature (Ta) closer to the Ta of the reverse primers (Matsunaga et al., 1999). The primers were synthesized by Integrated DNA Technologies, Pte. Ltd. (Singapore).

Table 1 Design of primer in duplex PCR

Primer	Description	GenBank accession no.	Sequence (5'-3')	Fragment size (bp)	Nucleotides in genome	Tm (°C)
MCF_2	Common forward primer	AY235570.1 (chicken), GU905063 (turkey), HQ699486 (duck), AY521209 (goose)	tggagaacatgctggtgatgacggccatgcccaagaac	n/a	457-494	73.0
CaR	Common adapter reverse primer	Hanapi et al. (2015)	*ccttccttccttccttcc*	n/a	n/a	52.1
MAviR	Avian-specific reverse primer	AY235570.1 (chicken), GU90506 3(turkey), HQ699486 (duck), AY521209 (goose)	tgtccatgtggcggacgatgc	191	630-650	62.3
MChiR	Chicken-specific	AY235570.1 (chicken)	atgctgtggtagtgcaacgca	304	743-763	59.8

	reverse primer					
MAviRa	Avian-specific adapter reverse primer	n/a	*ccttccttccttcc ttcc*tgtccatgtg gcggacgatgc	209	n/a	69.5
MChiRa	Chicken-specific adapter reverse primer	n/a	*ccttccttccttcc ttcc*atgctgtggt agtgcaacgca	322	n/a	69.5

Tm : Melting temperature as calculated by OligoAnalyzer 3.1. Actual anneling temperature was determined by the number of mismatch in the primer-template binding. n/a : Not applicable

Optimization of common primer-duplex PCR

The performance of the primers was first analyzed by assaying both conventional duplex PCR and common-primer duplex PCR. The 20 µL reaction mixture of conventional duplex PCR consisted of 1 × i-PCR-Red mix (i-DNA Biotechnology, Singapore), 0.25 mM of common forward primer (MCF2), 0.25 mM of each avian- and chicken-specific reverse primer (MAviR and MChiR), and 50 ng of chicken DNA. Meanwhile, amplification of the common-primer duplex PCR was accomplished in a final volume of 20 µL containing 1 × i-PCR-Red mix (i-DNA Biotechnology, Singapore), 0.25 mM of common forward primer (MCF2), 0.25 mM of common adapter reverse primer (CaR), 0.25 mM of each adapter reverse specific primers (MAviRa and MChiRa), and 50 ng of chicken DNA. The conventional PCR used the regular reverse primers while other applied adapter reverse primers.

The thermal cycling program of the conventional duplex PCR only contained one round of 35 cycles at the annealing temperature of 64 °C, while the common-primer duplex PCR comprised two rounds. The first round comprises 10 cycles, followed by the second round for 25 cycles, with an annealing temperature (Ta) of 50 to 68 °C for each round. Both assays started with an initial pre-denaturation step at 95 °C for 1 minute and 30 seconds, and each cycle includes 95 °C of DNA denaturation for 30 seconds, primer annealing for 30 seconds, and 72 °C of primer extension for 30 seconds.

The PCR products were analyzed by agarose (1.5% [w/v]) gel electrophoresis containing Red SafeTM (iNtRoN Biotechnologies) run in 1 × LB buffer. A 100-bp ladder, Kplus DNA ladder (GeneDirex®), was used as the DNA marker.

Primer specificity and sensitivity test

The specific MChiRa and MAviRa primers were tested against DNA extracts of eight different species (chicken, duck, turkey, cattle, deer, goat, pig, and rabbit) to detect any possible cross-reactivity that could affect the sensitivity of the assay. PCR amplification was performed using the optimized method above.

The sensitivity of the common-primer duplex assay was evaluated using the optimized Ta and PCR cycling parameters on 10-fold serial dilutions of DNA concentration starting from 0.1 to 0.0001 ng DNA to determine the limit of detection of the assay.

Meat identification

Common primer-duplex PCR was applied to the processed meat products in **Table 2**. Approximately 3 g of each product was used in the DNA extraction process, and 100 ng of each DNA sample was subjected to PCR analysis.

Table 2 Summarized results of duplex PCR to determine compliance to the labeling of meat product samples

Food category	Sample name/no.	Food type	Industrial meat processing method	Meat type/ Declared meat content	Avian detection	Chicken detection
Meats	C	Raw	None	Chicken	Detected	Detected
	D			Duck	Detected	Not detected
	T			Turkey	Detected	Not detected
	B			Cattle	Not detected	Not detected
	R			Deer	Not detected	Not detected
	G			Goat	Not detected	Not detected
	P			Pig	Not detected	Not detected
	O			Rabbit	Not detected	Not detected
Processed meat products	1	Rabbit burger	Minced, flavoured	Rabbit	Detected	Detected
	2	Beef sausage	Minced, flavoured, cooked	Cattle	Not detected	Not detected
	3	Beef sausage	Minced, flavoured, cooked	Cattle	Not detected	Not detected
	4	Mutton burger	Minced, flavoured, frozen	Goat	Not detected	Not detected
	5	Ostrich burger	Minced, flavoured, frozen	Ostrich	Detected	Not detected
	6	Deer burger	Minced, flavoured, frozen	Deer	Detected	Detected
	7	Deer burger	Minced, flavoured, frozen	Deer	Not detected	Not detected
	8	Deer burger	Minced, flavoured, frozen	Deer, cattle	Not detected	Not detected

9	Beef salami	Minced, flavoured, cooked	Cattle	Not detected	Not detected
10	Chicken burger	Minced, flavoured, frozen	Chicken	Not detected	Not detected
11	Chicken meatball	Minced, flavoured, frozen	Chicken	Detected	Not detected
12	Chicken roll	Minced, flavoured, cooked	Chicken	Detected	Not detected
13	Sandwich ham	Minced, flavoured, frozen	Pig	Not detected	Not detected
14	Lamb burger	Minced, flavoured, frozen	Sheep	Not detected	Not detected
15	Lamb burger	Minced, flavoured, frozen	Sheep	Detected	Detected
16	Pork meat ball	Minced, flavoured, cooked	Pig	Not detected	Not detected
17	Pork sausage	Minced, flavoured, cooked	Pig	Not detected	Not detected
18	Rabbit burger	Minced, flavoured, frozen	Rabbit, chicken	Detected	Detected
19	Turkey burger	Minced, flavoured, frozen	Turkey	Detected	Not detected
20	Turkey pastrami	Minced, flavoured, cooked	Turkey	Detected	Not detected
21	Turkey sandwich square	Minced, flavoured, cooked	Turkey	Not detected	Not detected
22	Chicken cocktail	Minced, flavoured, cooked	Chicken	Detected	Not detected
23	Chicken sausage	Minced, flavoured, cooked	Chicken	Detected	Not detected
24	Beef nuggets	Minced, flavoured, frozen	Cattle	Detected	Detected
25	Chicken ham	Minced, flavoured,	Chicken	Not detected	Not detected

		frozen			
26	Pork BBQ sausage	Minced, flavoured, cooked	Pig	Not detected	Not detected
27	Chicken cocktail sausage	Minced, flavoured, cooked	Chicken	Detected	Not detected
28	Minced pork	Minced, flavoured, cooked	Pig	Not detected	Not detected
29	Pork meat ball	Minced, flavoured, cooked	Pig	Not detected	Not detected
30	Chicken sandwich	Minced, flavoured, cooked	Chicken	Detected	Detected
31	Chicken BBQ sausage	Minced, flavoured, cooked	Chicken	Detected	Not detected
32	Cooked ham	Minced, cooked	Pig	Not detected	Not detected
33	Canned ham	Cured, sterilized	Pig	Not detected	Not detected
34	Chicken sandwich ham	Minced, flavoured	Pig	Not detected	Not detected
35	Pork luncheon meat	Minced, flavoured, cooked	Pig	Not detected	Not detected
36	Beef ball	Minced, flavoured, cooked	Cattle	Detected	Detected
37	Chicken luncheon	Minced, flavoured, cooked	Chicken	Detected	Not detected
38	Beef ball	Minced, flavoured, cooked	Cattle	Not detected	Not detected
39	Smoked chicken frankfurter	Minced, flavoured, cooked, smoked	Chicken	Detected	Not detected
40	Corned beef	Minced, flavoured, sterilized	Cattle	Not detected	Not detected
41	Corned beef	Minced, flavoured, sterilized	Cattle	Not detected	Not detected
42	Hamburger	Minced, flavoured	Pig, chicken	Detected	Detected

Results and Discussion

DNA quality

The main challenge in DNA extraction was the recovery of high-quality DNA from the vast array of complex food matrices. Meat products often contain both plant- and animal-based ingredients. Samples containing plant derivatives tend to be more challenging as plants contain high levels of polysaccharides, antioxidants, and many types of secondary metabolites that may affect DNA purity. A good extraction procedure for the isolation of DNA should yield an adequate quantity and reasonable DNA purity. Therefore, DNA from the meat products was isolated using the CTAB method, as described by Wu et al. (1995). The chemical components, i.e., sodium dodecyl sulfate (SDS), proteinase K, cetyltrimethylammonium bromide (CTAB), ethylenediaminetetraacetic acid (EDTA), and polyvinylpyrrolidone (PVP) in the CTAB lysis buffer, were estimated based on information from Besbes et al. (2011), depending on the nature of the meat products.

The concentrations of the DNA extracts were determined using a fluorescence-based (PicoGreen) method for obtaining a more precise concentration of DNA, especially from the processed meat samples that contain contaminants or other chemicals used in DNA extraction that are hardly removed. PicoGreen is a fluorochrome dye that is selectively bound to only double-stranded nucleic acids, excited by light at 485 nm, and emits fluorescence at 520 or 530 nm. From the assay, the concentration of DNA extracted from raw meats obtained was more than 500 ng/μL, while the purity of the DNA based on A260/A280 and A260/A230 absorbance ratios were between 1.73–1.98 and 1.82–2.15, indicating that the DNA was relatively free from protein contamination (Wilson & Walker, 2005; Result shown in supplementary data).

Primer design

In this study, primers were designed from a well-conserved region of the MC1R gene, which encodes for melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor) for coat color determination (Mohamad et al., 2013; Karsprzak-Filipek et al., 2020). The gene has been used in many studies for intraspecies differentiation of several animals, including humans, dogs, cats, pigs, horses, chickens, cattle, and sheep (Andersson, 2003; Evans et al., 2007; Kanthaswamy et al., 2012). It was also used in the interspecies differentiation of goat (Fontanesi et al., 2009), cattle (Russo et al., 2009), pig (Fajardo et al., 2008), sheep (Deng et al., 2009), elk (Lindquist et al., 2011), and domestic cat (Menotti-Raymond et al., 2009).

A 5′-CCTTCCTTCCTTCCCCCCC-3′ sequence of the common adapter reverse primer (CaR) was chosen, where cytosine (C) would never bind to thymine (T); hence, it would not complement each other to form a dimer or loop. It is also important that the sequence does not match any genome template, such as a microsatellite or short tandem repeats. The same sequence of CaR became the adapter attached at each of the 5′-end of the species-specific reverse primers to serve as a template for the CaR primer in the next PCR cycles. The adapter used in the system acts as a universal or common primer to lessen the significant differences in melting temperatures among the species-specific primers.

To assess the effectiveness of this assay for rapid detection, Ta between 50 and 68 °C were tested, and the PCR cycling parameters (total number of cycles and periods) were optimized. Amplification was performed in two rounds of cycling conditions. In the first ten cycles of amplification, specific adapter reverse primers (MAviRa and MChiRa) were allowed to anneal to the specific sequences of DNA samples at 64.0 °C. This step is considered as an enrichment of the target sequences. In the next 25 cycles, MCF2 and CaR were allowed to amplify the fragments from the templates synthesized in the first round PCR at a lower Ta

(53.0 °C).

Common primer-duplex PCR system

The amplification products of the common primer-duplex PCR and conventional duplex PCR assays are shown in **Figure 1**. The bands amplified by the common primer-duplex PCR system are more intense than those amplified by the conventional multiplex systems, suggesting that the amplification efficiency was increased by the adapter primers. Preliminarily, simplex PCRs were carried out on DNA extracted from raw meat to verify the specificity of the primers **(Figure 2a)**. Each set of primers was challenged in simplex PCR with non-target species to detect possible cross-reactions. In no case that cross-contamination was observed. Overall, the common primer-duplex PCR successfully identified the targeted 322 bp for chicken and 209 bp for all tested avian species (chicken, duck, and turkey) of the MC1R gene **(Figure 2b)**. The application of avian universal primer in meat detection in this study may provide double validations, which have not been reported by other researchers.

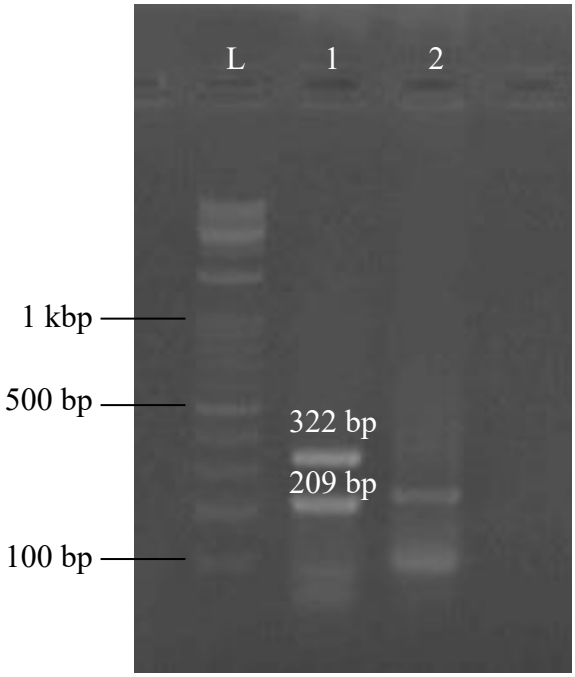

Figure 1 Comparison of PCR products amplified with common primer duplex PCR (lane 1) and conventional duplex PCR (lane 2). L, Kplus DNA ladder (GeneDirex®)

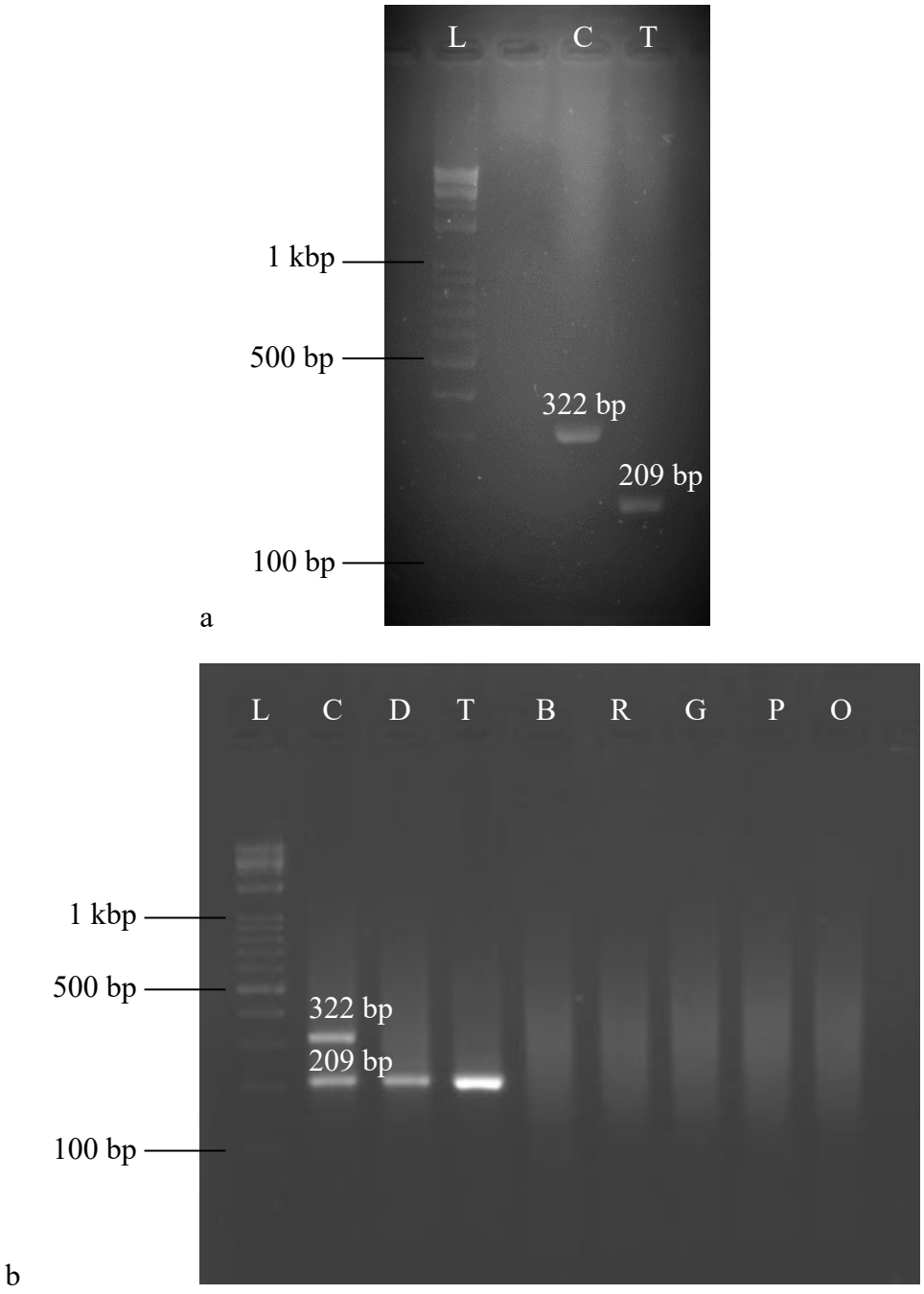

Figure 2 PCR amplification of chicken specific primer (MChiR) and avian universal adapter primer (MAviR). (a) Simplex amplification of chicken (C) and avian (T;turkey), and (b) specificity test. C, chicken; D, duck; T, turkey; B, cattle; R, deer; G, goat; P, pig; O, rabbit; L, Kplus DNA ladder (GeneDirex®)

This study also determined the limit of detection (LOD), which is defined as the minimum amount of target DNA sequence that can be detected in a sample (Hanapi et al., 2015). **Figure 3** shows the results of PCR amplification from diluted DNA templates of 0.1, 0.01, 0.001, and 0.0001 ng. Lanes 1 to 4 show bands corresponding to avian and chicken, indicating the detection limits at 0.01 ng. This study produced a better sensitivity than that reported by Bai et al. (2009), who obtained a sensitivity of 0.1 ng DNA, which was claimed to be a sufficient level to detect the presence of food fraud in commercial meat products. Other

common primer PCR systems, such as common single primer multiplex PCR, also showed a detection limit of 0.1 ng DNA of minced meats (Xu et al., 2008). For future studies, primer binding sites can be further manipulated to amplify specific fragments of less than 200 bp in length to be used in real-time PCR, where much lower LOD can be achieved. This is also important to increase the chances of achieving amplification from a smaller allele from a highly degraded DNA sample. Ultimately, the efficiency in the detection of meat contaminants can be improved in future studies.

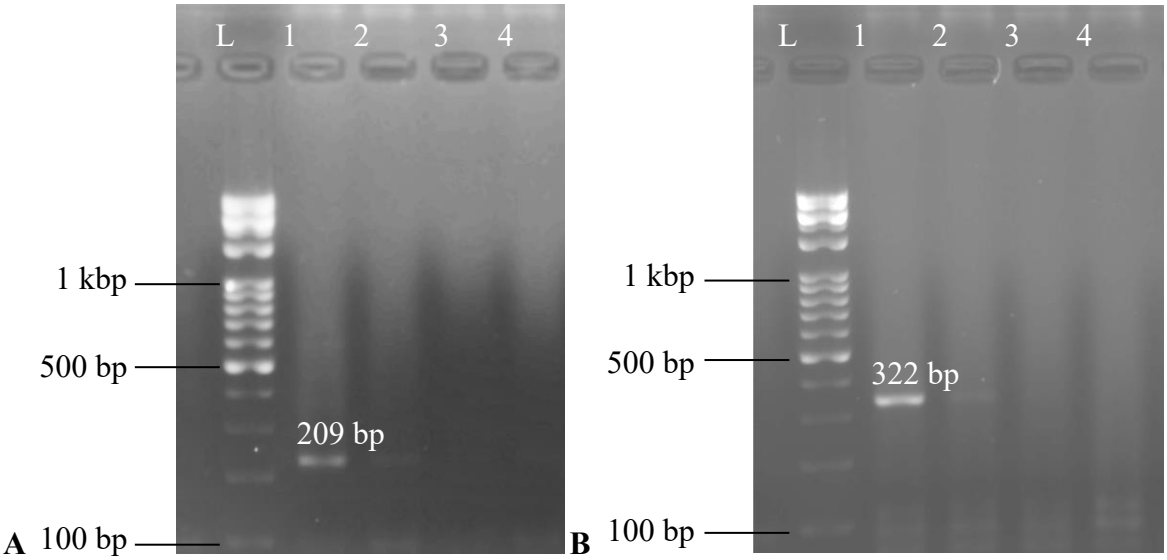

Figure 3 Determination of detection limit of (A) avian specific primer, MAviR; (B) chicken specific primer, MChiR. Serial dilutions of chicken DNA. Lane 1, 0.1 ng; lane 2, 0.01 ng; lane 3, 0.001 ng; lane 4, 0.0001 ng; L, Kplus DNA ladder (GeneDirex®)

Avian and chicken detection in commercial samples

The model was applied to commercial meat product samples to verify labeling compliance and evaluate the possible existence of fraudulent practices. Of the 42 food products comprising 22 samples identified as avian meat-based samples, 17 were declared to contain avian meat, and 5 were tested as undeclared. **Table 2** compiles the results of the 42 food products, indicating avian content authenticated in 19 out of 42 meat product samples examined, reflecting 45% of the samples. The MAviRa primer showed positive amplification of a 209-bp fragment in all avian samples except for samples 10 (chicken), 21 (turkey), and 25 (chicken).

Additionally, chicken DNA was not detected by the MChiRa primer in eight processed meat samples declared to contain chicken meat (samples 11, 12, 22, 23, 27, 31, 37, and 39) due to their larger fragment size than that of the avian-specific primer. The absence of the expected PCR band in those samples might be due to intense food processing or long storage, resulting in detection failure due to DNA fragmentation, which requires a more sensitive detection procedure that targets much smaller fragments. Although DNA exhibits fairly high thermal stability, it is well-known that intense overheating and freezing may cause severe DNA degradation (Mohamad et al., 2015; Zhao et al., 2018).

The qualitative determination also confirmed the presence of declared chicken content in the rabbit burger (sample 18) and hamburger (sample 42). On the contrary, the potential undeclared presence of chicken and avian in meat products was observed in five cases in this study, i.e., the rabbit burger (sample 1), deer burger (sample 6), lamb burger (sample 15), beef nugget (sample 24), and beef ball (sample 36). The low cost of chicken compared to other meat groups may be the reason for it being partly substituted with other expensive meat to reduce

cost. The wide availability and versatility of chicken meat to mimic the component and structure integrity of real processed meat (Lee et al., 2018) could also be the reason for its use in partial substitution of the actual meat content. Nevertheless, cross-contamination might also occur during food manufacturing in the factory. This may not reflect a good manufacturing practice, which is an important factor in guaranteeing the quality and cleanliness of products.

Conclusion

In summary, the developed common primer-duplex PCR system in this study offers the advantages of being cheap, rapid, reliable, sensitive, and useful for routine analysis of large numbers of samples, especially by local authorities in inspection programs to enforce labeling regulation of meat products. Both universal and species-specific detection of avian and chicken provided by the PCR system can potentially resolve chicken meat fraud and incorrect labeling issues in processed meat products that are available in the market. However, the use of these primers in highly processed or cooked products is restricted, particularly concerning the chicken-specific adapter primer. Hence, chicken-specific primers may be redesigned to target smaller DNA fragments less than 300 bp. This will provide a more sensitive detection for highly degraded DNA samples of highly processed meat products.

Ethics Statement

The research does not require research ethics approval.

Authors Contribution

"Writing – Original draft preparation, Hanapi, U. K.; Writing – Review and editing, Mohd Desa, M. N., Mohamad, N. A. & Khairil Mokhtar, N. F."

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Conflict of interests

All authors declare no conflict of interest.

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