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Developing a Multiplex PCR Technique for the Identification of Halal and Non-Halal Meats

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Abstract:

Food safety is a major concern in most Muslim countries (e.g., Iran), where halal and non-halal meat adulteration is believed to cause significant social and health risks. Thus, developing a valid and reliable detection method to distinguish between halal and non-halal meats in processed foods is crucial, particularly for small-scale laboratories. In this regard, this current work aimed at designing and optimizing species-specific primers to identify halal and non-halal meats in processed foods with the use of the NADH Dehydrogenase and ATP Synthase genes via simplex and multiplex PCR assays. The results depicted that the DNA extracted from processed beef, poultry, and pork could be effectively amplified using the NADH Dehydrogenase and ATP Synthase primers, producing amplicon bands that were visible and consistent with the expected size in both simplex and multiplex PCR experiments. Despite the fact that non-specific bands were observed in several primer sets, the primary target bands remained distinct and easily identifiable. The existence of such primers is expected to promote the efficiency of halal food authentication, particularly in small-scale laboratories in rural regions of Iran.

INTRODUCTION

Iran has a large Muslim population and is globally ranked among the countries with the highest Muslim community. As such, observance of halal dietary regulations is a fundamental obligation for Muslims, who label certain food products (e.g., porcine-derived foods) as non-halal (haram)—prohibited for consumption (1). As a consequence, the availability of halal food is a critical concern in countries with large Muslim populations, such as Iran (2).

Meat is considered the principal source of protein in Iran, with poultry and beef being the most popular varieties (3). As declared by the Iranian Ministry of Agriculture, in the year 2024, the average annual consumption of poultry meat and red meat per inhabitant was respectively 27 kg and 14 kg. With the availability of several processed poultry products such as sausages, nuggets, chicken and beef meatballs, and meat floss, there is always the possibility that the halal meat products may have non-halal meat mixed during processing (4). Notably, among the many available products, halal meats, such as beef and lamb, are more susceptible to adulteration with pork by dint of analogous color and texture profiles (5). The mixing of non-halal and halal meats in processed products can result in consumer dissatisfaction, social and religious concerns, and potential health-related risks. Therefore, developing detection technologies capable of identifying porcine adulteration in processed foods is fundamental to ensure the integrity of halal meat products (5, 6).

To date, a number of analytical techniques have been developed and proposed for detecting pork adulteration in food products, videlicet Enzyme-Linked Immunosorbent Assay (ELISA) (7, 8, 9), high-performance liquid Chromatography-Mass Spectrometry (HPLC-MS) (10), Loop-Mediated Isothermal Amplification (LAMP) (11, 12), conventional Polymerase Chain Reaction (PCR) (13), Real-Time PCR (RT-PCR) (14, 15, 16), and gold nanoparticle-based assays (17, 18). Among these methods, conventional PCR is found to be a relatively easy-to-use and accessible technique, and this has made it a good candidate for use in small-scale laboratories in Iran. Not to mention that the PCR-based detection method enjoys high sensitivity that enables

the identification of porcine DNA contamination at concentrations as low as nanogram levels.

In standard PCR-oriented detection methods, molecular markers have been developed to recognize pork adulteration in processed products by targeting specific gene sequences. Typically, mitochondrial genes such as 12S rRNA, 16S rRNA, cytochrome b (cyt b), and D-loop have so far been used to develop these markers (19, 20, 21). Nonetheless, alternative genes, viz NADH Dehydrogenase and ATP Synthase have marginally been recruited to date (22). These genes encode essential enzymes involved in the mitochondrial respiratory chain, with NADH Dehydrogenase facilitating electron transfer and ATP Synthase catalyzing the ATP synthesis from ADP (23). These housekeeping genes play a pivotal role in balancing cellular respiration and showing high sequence conservation across different animal species. This has made these genes promising candidates for molecular marker development (23, 24).

Previous research (24) demonstrated the efficacy of a multiplex PCR assay that used the NADH Dehydrogenase gene as a molecular marker to identify meat from four different animal species (pig, ruminant, bird, and rabbit). Nonetheless, no previous work has been reported to have applied the ATP Synthase gene in this context. Noteworthy, the aforementioned study (24) focused on raw meat samples, whereas the present investigation aimed at developing and validating species-specific primers for differentiating between halal and non-halal meats in processed food products using simplex and multiplex PCR approaches. For this purpose, the NADH Dehydrogenase and ATP Synthase genes were considered molecular markers. The development of these primers is expected to enhance the precision and efficiency of halal food authentication, more specifically in small-scale Iranian laboratories, where access to advanced molecular diagnostic tools might be limited.

MATERIALS AND METHODS

Food samples

A total of seven processed food products, namely sausage, nuggets, meatballs, rendang, dendeng, floss, and skin crackers, were purchased from local markets and online retailers (see Table 1). The primary ingredients of these products included pork, beef, and chicken. The samples were stored at -20 ℃ in a refrigerated environment to protect their integrity and prevent degradation until further analysis.

Genomic DNA Extraction

A modified protocol (25) was used to isolate genomic DNA from the processed food samples. In brief, a sterile mixer was used to homogenize 0.1 g of each sample in 500 µL of lysis buffer (250 µL of 10%

Table 1. List of processed foods used in this study

Sample code	Processed food sample	Processed food source
1	Beef rendang	Local market
2	Beef dendeng	Local market
3	Beef floss	Online shop
4	Beef sausage	Local market
5	Beef meatball	Local market
6	Chicken floss	Online shop
7	Chicken meatball	Local market
8	Chicken nugget	Local market
9	Chicken sausage	Local market
10	Pork sausage	Online shop
11	Pork floss	Online shop
12	Pork meatball	Online shop
13	Pork dendeng	Online shop
14	Pork rendang	Online shop
15	Pork skin cracker	Online shop

sodium dodecyl sulfate (SDS) and 250 µL of sodium chloride-tris-EDTA (STE)). The obtained mixture was transferred to a 2 mL microtube and incubated at 58°C for 1 hour with 20 µL of Proteinase K, with periodic mixing every 10 minutes ensuring uniform digestion. Afterwards, the mixture was subjected to two consecutive extractions with 800 µL and 600 µL of chloroform:isoamyl alcohol solution (24:1), respectively, with centrifugation at 12,000 rpm for 10 minutes at 20°C between each extraction. Next, the acquired supernatant was precipitated with 3 M sodium acetate (pH 5.2) and cold isopropanol, followed by incubation at -20 °C for 1 hour and centrifugation at 12,000 rpm for 10 minutes at 20°C. The DNA pellet was washed with 70% (v/v) ethanol, dried overnight at room temperature, and reconstituted in 100 µL of TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA). The DNA concentration was adjusted to 20 ng/µL for subsequent amplification. The quality and purity of the isolated DNA were then evaluated with the use of the NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA), with high-quality DNA exhibiting an A260/A280 ratio between 1.8 and 2.0.

Primer Designs

Gene sequences of the NADH Dehydrogenase and ATP Synthase genes from bovine (Bos taurus), chicken (Gallus gallus), and pig (Sus scrofa) were retrieved from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/gene/). The retrieved sequences were subsequently used to design specific primers using the Primer3Plus software (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (26). To ensure specificity, the designed primers were then evaluated against reference sequences (RefSeq mRNA) from cattle, chickens, and pigs utilizing the Primer-BLAST tool(https://www.ncbi.nlm.nih.gov/tools/primer-blast/) on the NCBI platform. Confirmed by BLAST analysis, the primer design was then optimized to ensure a single annealing site for each primer (see Table 2 for the acquired primer sequences).

Simplex PCR assay

To evaluate the specificity and efficacy of the newly designed primers, a simplex PCR assay was carried out using the primers that targeted the NADH Dehydrogenase and ATP Synthase genes. The PCR reaction mixture comprised 2 µL of 20 ng/µL DNA template, 5 µL of 2x MyTaq HS (Bioline, UK), 0.5 µL

Table 2. Primer pairs that were used in this study

Gene	NCBI Accessions Number	Species	Primer (5'-3')	Ta* (°C)	Product Size (bp)
NADH Dehydrogenase	NM_174565.3	Bovine	F: GGAGCTGGAGGAGCCTTATT R: TTTCCAACAGGCTTTCGATT	58	290
	XM_046907522.1	Chicken	F: CTGAAGACCCACAGCAGACA R: GAGGACGCTGTCCTTCACTC	58	405
	NM_001243796.1	Pig	F: CTGCCTGTGAGGAAGGAAAG R: AGGATTTCGGCCACATACTG	58	113
ATP Synthase	NM_175796.3	Bovine	F: AGCCCATGGTGGTTACTCTG R: TTGCCACAGCTTCTTCAATG	58	881
	NM_001031391.3	Chicken	F: AGGGCAACGATTTGTACCAC R: GGTCAGCTTGTCCTCCTCAG	58	635
	NM_004046.6	Pig	F: TCCAGAAATGCTTTGGGTTC R: ATTGGCACCAAGCTATCCAC	58	542

*Ta: temperature annealing

each of forward and reverse primers at a concentration of 10 µM, and sterile ddH2O, in a total reaction volume of 10 µL. The PCR amplification was implemented in a Thermocycler (Biorad, USA) with the use of a thermal cycling profile consisting of an initial denaturation at 95°C for 5 minutes, followed by 32 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 1 minute, and extension at 72 °C for 1 minute. The final extension was executed at 72 °C for 5 minutes. The resulting PCR products were separated by 1% agarose gel electrophoresis in a 1x TBE buffer at 90 V for 60 minutes. Afterwards, the gel was stained with a 10 mg/ml solution and was visualized under ultraviolet light using a UV transilluminator (Biorad, USA). For comparison, a previously reported primer (27).

Multiplex PCR assay

To simulate the detection of adulteration between non-halal meat (pork) and halal meats (beef and poultry) in processed food products, a multiplex PCR assay was conducted by combining DNA templates from processed meat products containing pig with those from products containing beef or chicken DNA in a 1:1 ratio (see Table 3). Specifically, 1 µL of DNA template from a beef sausage sample was mixed with 1 µL of DNA template from a pork sausage sample, and subsequently amplified in a total reaction volume of 10 µL, comprising 5 µL of 2x MyTaq HS (Bioline, UK), 0.5 µL each of forward and reverse primers at a concentration of 10 µM, targeting the bovine, poultry, and pig NADH Dehydrogenase and ATP Synthase genes in a 1:1:1 ratio, and sterile ddH2O. The thermal cycling profile and electrophoresis procedure were identical to those employed in the simplex PCR experiment. Furthermore, a multiplex PCR experiment was also conducted using a previously reported primer (27) as a control, following the same PCR and electrophoresis protocols as the newly developed primer.

RESULT

DNA Concentrations and Purities

The DNA concentration and purity measurements obtained by the NanoDrop 2000 Spectrophotometer (ThermoScientific, USA) are shown in Table 4. While the values of DNA purity ranged from 1.38 (pork rendang) to 2.02 (chicken nugget), the DNA concentrations extracted from processed food samples varied from 25.9 ng/µL (chicken floss) to 669.1 ng/µL (beef dendeng). Notably, the average DNA concentration and purity values for the entire dataset were 230.93 ng/µL and 1.77, respectively. Although some samples exhibited DNA purity values below the optimal threshold of 1.80, the repeated extraction process that used chloroform:isoamyl alcohol (24:1) mixture significantly reduced contamination by proteins and other impurities, as evidenced by the median DNA concentrations. The combination of chloroform and isoamyl alcohol was found to be essential for the degradation of protein and prevention of emulsion formation, which resulted in the enhancement of DNA precipitation and improvement of the overall quality of the extracted DNA (28).

The simplex PCR results using NADH Dehydrogenase primers yielded distinct and well-defined amplicon bands (see Figure 1). The amplicon sizes obtained from each processed product derived from beef, poultry, and pork corresponded to the expected target sizes of 290, 405, and 113 base pairs, respectively. Nevertheless, the primer obtained from the bovine sequence showed non-specific amplicon bands that were larger than the intended size, which were difficult to eliminate. Yet, as the primary amplicon band for this primer was visible and distinct from the non-specific bands, this result is still considered acceptable.

Consistent with the results obtained using NADH Dehydrogenase primers, the simplex PCR results

Table 3. DNA combination from processed food for multiplex PCR assay in this study

Sample Code	Food Processed Combination
A	Beef and pork rendang
B	Beef and pork dendeng
C	Beef and pork floss
D	Beef and pork sausage
E	Beef and pork meatball
F	Chicken and pork floss
G	Chicken and pork meatball
H	Chicken and pork sausage

Table 4. DNA concentration and purity extracted from processed foods in this study.

Sample code	Processed food sample	DNA concentration (ng/µL)	DNA purity (A_{260}/A_{280})
1	Beef rendang	478.4	1.80
2	Beef dendeng	669.1	1.77
3	Beef floss	444.8	1.93
4	Beef sausage	138.4	1.94
5	Beef meatball	314.0	1.87
6	Chicken floss	25.9	1.57
7	Chicken meatball	416.4	1.67
8	Chicken nugget	121.5	2.02
9	Chicken sausage	41.6	1.88
10	Pork sausage	186.6	1.71
11	Pork floss	41.0	1.50
12	Pork meatball	227.0	1.92
13	Pork dendeng	47.2	1.96
14	Pork rendang	31.3	1.38
15	Pork skin cracker	280.8	1.59
	Average	**230.93**	**1.77**

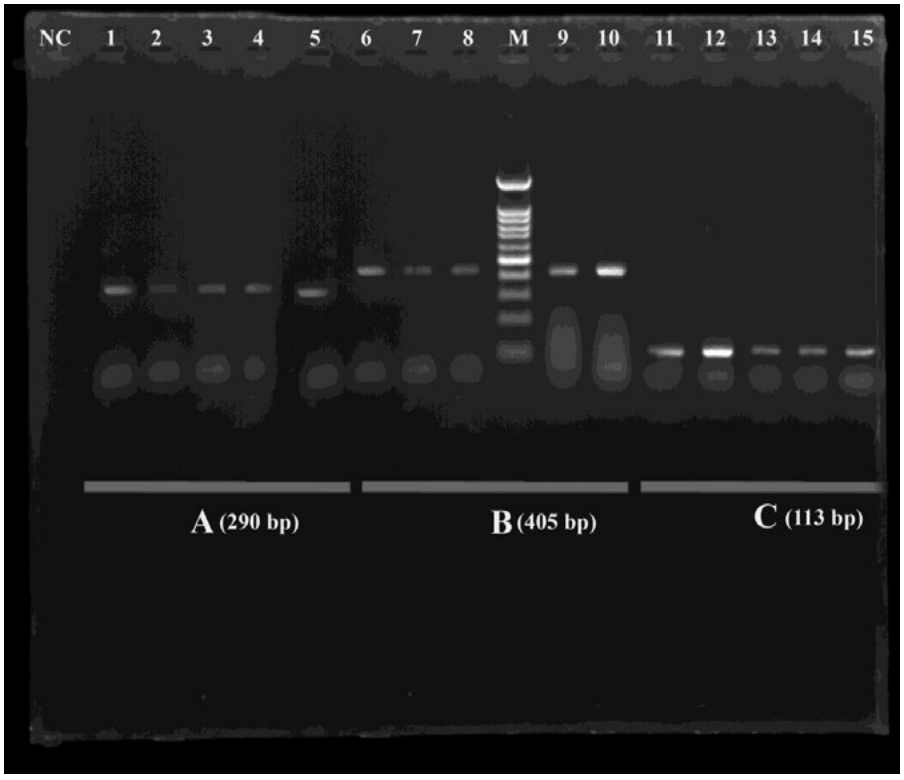

Fig1. Identification of simplex PCR results from: (A) 290 bp of beef, (B) 405bp of poultry, and (C) 113bp of pork, generated with NADH Dehydrogenase primers. M: 100 basis points DNA ladder, 1-15: enumeration of processed meals as delineated in Table 1.

employing ATP Synthase primers also produced visible amplicon bands (see Figure 2). The amplicon sizes achieved from each processed product derived from beef, poultry, and pork corresponded to the expected target sizes of 881, 635, and 542 base pairs, respectively. It should be noted that despite the presence of non-specific bands, the principal amplicon band for each primer remained distinct and well-defined, which is consistent with the results obtained using NADH Dehydrogenase primers for bovine, pig, and chicken.

In contrast to the results obtained with the NADH Dehydrogenase and ATP Synthase primers, the simplex PCR assay that used primers from earlier work (27) also yielded visible amplicons (see Figure 3). The amplicon sizes obtained from each processed product derived from beef, poultry, and pork corresponded to the expected target sizes of 274, 227, and 398 base pairs, respectively. Notably, the use of these primers led to a considerable reduction in non-specific amplicon bands, with all primers from bovine, chicken, and pig producing a single, distinct, and readily identifiable single band.

Multiplex PCR Assay

The results from multiplex PCR that used DNA mixtures from processed pork mixed with beef or chicken revealed the existence of clearly visible amplicon bands that matched the expected sizes for NADH Dehydrogenase, ATP Synthase, and Matsunaga et al. (1999) primers (see Figure 4). It is noteworthy that the genetic material from each component in the mixture was successfully identified and isolated, showing no evidence of interference from non-specific amplicons. The findings unraveled that the multiplex PCR assay is capable of simultaneously detecting and identifying the genetic material from diverse sources, thereby making the detection of any impurities in processed meat products easier.

This study employed a multiplex PCR approach to simulate real-world scenarios of halal detection in processed food products, including adulteration with non-halal components. A hypothetical example of a beef meatball from brand A suspected of containing pork was used to demonstrate the feasibility of the method. The approach involved isolating and amplifying DNA from the meatball using primer pairs targeting NADH dehydrogenase and ATP synthase genes in both bovine and porcine samples. To evaluate the efficacy of the primer mixture, DNA mixtures from bovine or chicken products were combined with DNA from pork products and amplified using a multiplex PCR assay with NADH dehydrogenase, ATP Synthase, and previously established primer pairs (27) for both bovine or chicken and pork. The results demonstrated that the primer mixture could successfully differentiate between halal (beef or chicken) and non-halal (pork)

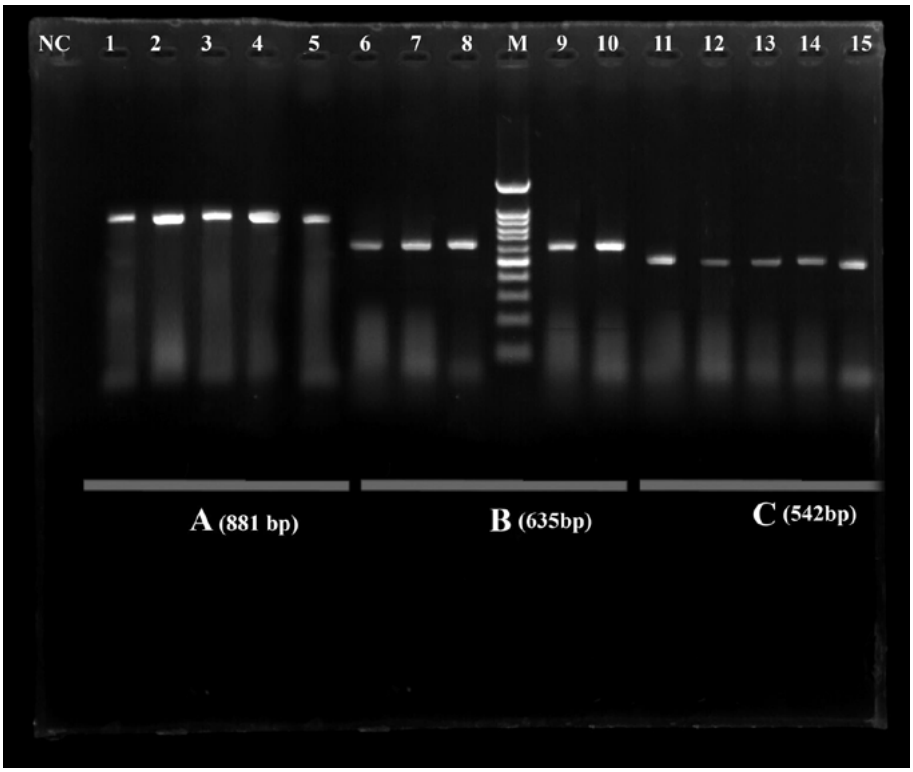

Fig 2. Characterization of simplex PCR results from: (A) beef, (B) chicken, and (C) pork, generated with ATP Synthase primers. M: 100 basis points DNA ladder, 1-15: enumeration of processed meals as delineated in Table 1.

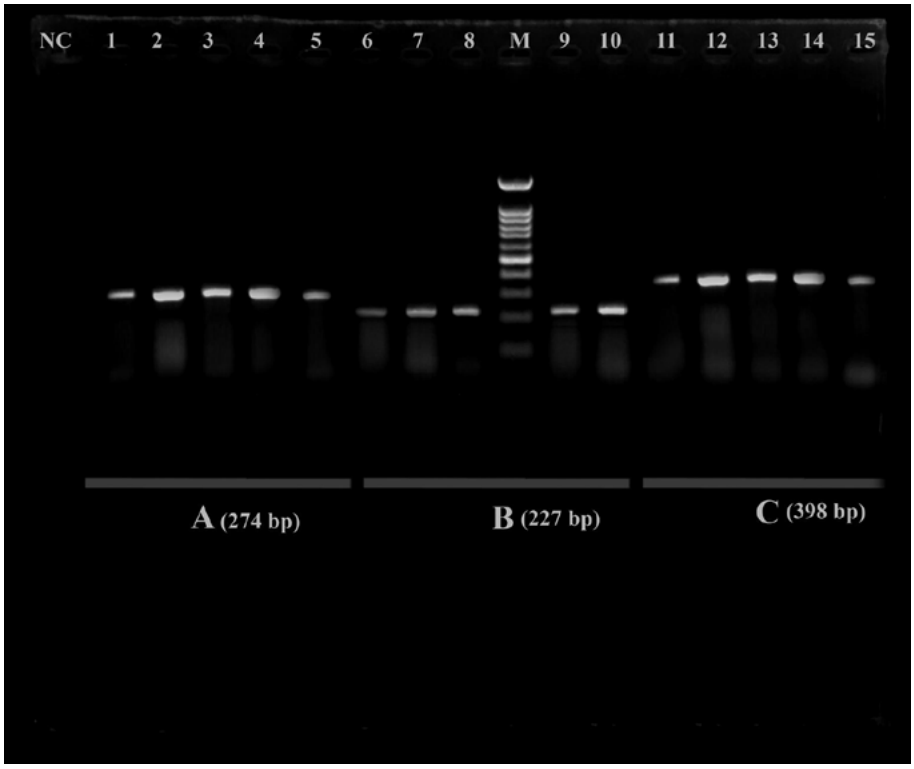

Fig3. Identification of simplex PCR results from (A) beef, (B) chicken, and (C) pork, generated with primers from prior research (27). M: 100 basis points DNA ladder, 1-15: enumeration of processed meals as delineated in Table 1.

DNA based on amplicon band sizes as separated in agarose gel, indicating the potential of this approach for detecting adulteration in processed food products.

DISCUSSION

Safeguarding food safety and security is a notable global concern, with food adulteration as a significant threat to public health. In Iran, food adulteration is still a persistent issue despite the establishment of the National Agency for Drugs and Foods Control (BPOM), which is responsible to regulate the food industry and oversee halal product certification (29). It is worth mentioning that small and medium-sized enterprises (SMEs) and traditional market sellers are more inclined to be engaged in meat adulteration (30). As such, developing a reliable detection technology that can recognize halal from non-halal meat in processed foods is essential to reduce such risks.

As an Islamic nation, the establishment of small-scale labs capable of conducting halal food detection in rural areas is essential to bolster the food safety program in Iran. Conventional PCR is a technology appropriate for use in small-scale labs in Iran due to its speed, sensitivity, accuracy, affordability, and ability to identify DNA contamination in minimal quantities, down to nanogram levels (31, 32).

In this study, the novel primers derived from the NADH dehydrogenase and ATP synthase genes effectively amplified all samples, producing clear and distinct amplicons that matched the target size in both simplex and multiplex assays. The consistency of amplicon size between the two PCR procedures was also observed. Furthermore, the previously developed primers (27, 32) targeting the cytochrome b gene also showed high amplification efficiency, yielding clear and visible bands of the predicted size.

The original primer set that was developed in this study could successfully amplify the target genes but also yielded non-specific amplicon bands. Multiplex PCR is a simultaneous amplification procedure of several DNA targets inside a single reaction, and primer design is a vital and fundamental phase in the construction of a multiplex PCR test (17, 31, 32). In the design process, several parameters, namely primer length, GC content, melting temperature, and potential secondary structures (e.g., such as dimers and hairpin loops) must be computed meticulously to ensure specific and efficient amplification of the target genes. Furthermore, other factors, such as PCR conditions, DNA template concentration, and PCR cocktail composition, that can influence multiplex PCR performance, must be thoroughly evaluated and optimized during the development process.

The annealing temperatures for the NADH dehydrogenase and ATP synthase primers, specifically 58 °C, were determined through prior

optimization studies. Yet, increasing the annealing temperature for the bovine NADH Dehydrogenase and pig ATP synthase primers to 58 °C or higher may help eliminate non-specific amplicon bands. Nevertheless, this approach may compromise the amplification of other primers, resulting in reduced or absent amplicon bands due to suboptimal annealing conditions. Therefore, the annealing temperature was carefully selected to make sure that all primers were able to produce distinct and visible amplicon bands. This optimization procedure is a critical constraint in multiplex PCR assays, as it is challenging to simultaneously optimize the melting, annealing, and elongation temperatures for multiple primers while minimizing the formation of secondary structures, primer-dimers, and non-specific amplicon bands (34). Notably, as shown in the current work, if the primary target band remains detectable and visible, the existence of non-specific bands may be tolerated.

The novel primers developed in this study have the potential to improve the halal detection technique using traditional PCR, which has been previously employed with various genes, including 12S rRNA, 16S rRNA, cyt b, and D-loop. The ability of these primers to amplify small amounts of DNA extracted from processed foods makes them a valuable tool for halal identification, thereby enhancing food safety programs in Iran. Furthermore, the newly designed primers may be adapted for use in a multiplex end-point PCR approach, as demonstrated in previous studies (27, 34), by utilizing a single universal forward primer in combination with multiple species-specific reverse primers, which could help minimize production costs.

CONCLUSION

The novel primer sets developed in this study, targeting the ATP synthase and NADH Dehydrogenase genes, demonstrated high efficacy in amplifying DNA from all processed food samples in both simplex and multiplex PCR assays. The generated amplicon bands were visible and corresponded to the expected size, comparable to primers from a previous investigation. However, a limitation of this study was the observation of non-specific bands in the bovine NADH Dehydrogenase and pig ATP Synthase primers. Nevertheless, the presence of these non-specific bands was deemed acceptable, as the primary target band remained distinct and visible. The newly developed primers in this study have the potential to improve the halal detection technique using traditional PCR, which has been established to date, and could serve as a valuable tool for halal detection, thereby supporting food safety programs in Iran.

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Authors' contributions

Conceptualization: Romina HosseinZadeh, Methodology; Romina HosseinZadeh and Zahra Sorkhou, Investigation: Romina HosseinZadeh and Zahra Sorkhou, Writing-review and editing: Romina HosseinZadeh and Zahra Sorkhou. All authors reviewed the manuscript.

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Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent to publication

Not applicable.

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