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Detection of Minced Red Meat Mixing Adulteration via Molecular and Histological Techniques in Mashhad, Iran

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Abstract

Background: Meat is an important source of protein and due to its high economic value, there is a possibility of using other animals' tissues and cross-species adulteration to reduce its price. The meat industry has the highest potential for adulteration among food groups, since the raw materials are not identifiable after mixing, making the detection of food adulteration a necessity.

Methods: In this study, 14 samples of minced red meat from 14 butcher shops in Mashhad were randomly selected, collected, and analyzed using histological and molecular techniques. For histological analysis, the samples were prepared according to the usual methods and the prepared sections were stained using conventional and tissue-specific staining. Molecular analysis was performed using the Real-time PCR technique. The data were analyzed using Rotor-Gene Q software 2.3.5.

Results: The histological analysis confirmed the presence of gizzard and chicken skin in addition to skeletal muscle, smooth muscle, and adipose tissue in the minced red meat samples. Furthermore, the molecular analysis confirmed the use of chicken meat or chicken waste in a number of samples by confirming chicken DNA.

Conclusion: Histological and molecular techniques confirmed the presence of chicken tissues in some minced red meat samples, which may have been used to reduce the price of minced red meat but is considered as food adulteration.

Keywords: Red meat, White meat, Food adulteration, Histology, Molecular method

Introduction

Meat is considered one of the most important foods that can provide the protein needed by the body. Besides, the meat industry is the one with a higher potential of adulteration compared to different food groups, because the raw materials after being mixed and getting uniform are not recognizable (1). The presence of unauthorized tissues in minced meat is a real problem in its supply. Therefore, using accurate techniques to identify unauthorized tissues in minced meat is

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essential and helps determine the quality of this type of product (2). In addition to tissue sections and histological images that are widely used in the detection of adulteration in minced meat, identifying the authenticity of meat is also an important issue that can contribute to controlling the quality of meat (3).

Immunological and electrophoresis techniques were used in the past to detect proteins of different species in meat. These techniques were complex and also led to a possibility of cross-reactions between closely related species. Accordingly, these techniques were replaced by DNA-based methods for food authentication. DNA has fixed properties that do not change with heat, pressure, and chemicals, and the sequence of fragments in a particular tissue can be identified in a particular animal (4). DNA-based polymerase chain reaction (PCR) is a simple technique that, in addition to saving time, is a sensitive method that can easily identify different species (5). In addition to the PCR technique, a newer method called the Real-time PCR method has been developed based on the Taq Man probe in which in addition to a specific primer pair, a specific probe is used which increases the specificity and sensitivity of the test (6). Real-time PCR is superior to PCR in that it can detect very small amounts of DNA of the target species in a particular mixture (7, 8).

Conventional chemical tests of meat quality control, if used in conjunction with histological methods, have more accurate applications to identify unauthorized tissues in meat products and make the detection of adulteration more reliable and easier (9).

This study aimed to investigate the presence or absence of chicken meat and chicken waste in bulk minced meat as a type of food adulteration. To this end, two histological and molecular techniques were used for the first time in this study. Previous studies have used either molecular methods or histological methods, and no information has been found on the mixed-use of molecular and histological methods.

Methods

The present study was conducted as part of a research project required for a student dissertation approved by Ferdowsi University of Mashhad with a code of 48461/3. In this study, the city of Mashhad was divided into fourteen geographical areas. The number of stores in each area was counted and they were numbered sequentially. Then, a number showing the store number in each area was randomly selected. Subsequently, fourteen red meat (butcher) stores in the city of Mashhad were selected and 14 samples including minced beef, or mutton, or a mixture of minced beef and mutton, weighing approximately 200 grams per sample were prepared. The collected samples were transferred to the histology laboratory of the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, and their specifications were recorded to perform the necessary tests. To this end, all samples were numbered and divided into two groups.

The first group selected for histological tests was fixed in 10% buffer formalin. Then, the tissue preparation steps including dehydration with alcohol, clarification with xylene, and impregnation with paraffin (made by Merck Germany) were performed by a preparation device. Afterward, the samples were molded in Lockhart molds with molten paraffin and paraffin blocks were prepared. To perform histological studies, each sample was divided into three sub-samples and then the blocks were cut into 5 μm-thick sections by a semi-automatic microtome machine (Leica Model). Accordingly, 3 sections were prepared for each sub-sample. Finally, a total of 126 tissue slides were prepared and stained with three types of dyes: hematoxylin and eosin, Masson-trichrome, and PAS-alcian blue (Merck Germany) and analyzed by Olympus microscope (U-TVO 63XC, Japan).

During the staining process, the prepared sections were placed in special staining baskets and for removing paraffin and staining, they were placed respectively in xylene 1, and 2 , pure alcohol, 96%, 80% and 70% alcohol. Then, the sections were placed into a hematoxylin dye solution. After immersing the baskets containing tissue sections in an alcohol-acid solution for a few seconds, they were placed in the eosin dye solution and then 70-80-96% and pure alcohol. In Masson's

trichrome staining, after the paraffinization and alcohol hydration steps, fixation was performed with Bowen's solution overnight at room temperature. The samples were then immersed in Weigert solution for 15 minutes, Birlich-Scarlett-Fuchsin acid solution for 5 minutes, 5% phospho-molybdic-phospho-tungstic acid solution for 15 minutes, 2% light green solution for 1 minute, and 1% glacial acetic acid solution for 5 minutes. In each of these steps, the samples were rinsed with water and then dehydration was performed with alcohol. In PAS-alcian blue staining after paraffinization of the sections, the baskets were placed in PAS-alcian blue solution for 30 minutes and after rinsing in running water, they were placed in 0.5% periodic acid solution (for 5 minutes), Schiff solution (30 minutes), and hematoxylin (1-3 minutes) and washed with water at each stage. All times were measured and steps were performed according to protocols stated in the literature (10, 11).

The second group was selected to perform molecular tests, determine DNA, and identify the type of meat consumed in minced red meat. The selected samples were transferred to the molecular diagnostics laboratory fresh and without formalin storage and tested using Real-time PCR. The minced meat samples were identified by codes B1-B14. The samples were stored in the freezer at -20 °C as a control sample. DNA of minced red meat was extracted using a kit (Roche Co., Germany). First, according to the instructions provided by the kit manufacturer, 100 mg of the samples were homogenized and suspended in 200 μL PBS.

Then 200 µl tissue lubricating buffer and 40 µl proteinase K were added to the samples and incubated for 55 h at 55 °C. Then 100 µl isopropanol was added to the samples and mixed well and transferred to a tube filter and centrifuged at 8000 × g for 1 min. In the next step, the samples were washed twice with the help of a washing buffer, and at the end, a high-speed centrifuge was performed to dry the samples. Then, 200 µl of a solvent buffer, heated to 70 ° C, was added to the samples and by centrifugation, the DNA in the filter was dissolved and collected in the microtube. In the next step, the extracted DNA was amplified by Real-time PCR using primers and specific probes for each species. The Real-time PCR method uses hydrolyzable probes which are detected in each PCR cycle by releasing fluorescent light. The amount of fluorescence released indicates the number of mitochondrial DNA copies produced and determines the associated animal species. Specific oligonucleotide primers and probes of each species (Table 1) were purchased in pure and desalinated form from Metabion, Germany. In addition, pork and donkey meat detection primers (Kasman et al., 2009) and beef, mutton, and chicken meat detection primers (Switchi et al., 2007) were used. The primers were designed based on the mitochondrial cytochrome b gene (12-14).

Table 1. Arrangement of specific primers and probes for bovine, sheep, and gallus species

Bovine	Forward Primer	5´-CCCGATTCTTCGCTTTCCAT-3´
	Reverse Primer	5´-CTACGTCTGAGGAAATTCCTGTTG-3´
	Taq Man probe	5´-(FAM)-CATCATAGCAATTGCC-(NFQ)(MGB)-3´
Sheep	Forward Primer	5´-CCTTATTACACCATTAAAGACATCCTAGGT-3´
	Reverse Primer	5´-GGGTCTCCGAGTAAGTCAGGC-3´
	Taq Man probe	5´-(FAM)-ACTAATCCTCATCCTCATGC-(NFQ)(MGB)-3´
Gallus	Forward Primer	5´-TCTCACTTACACTACTTGCCACATCTT-3´
	Reverse Primer	5´-CGTGTGTGTCCTGTTTGGACTAG-3´
	Taq Man probe	5´-(FAM) –CACTGCAACCTACAGCCTCCGCATAAC-(BHQ)-3

The specificity and sensitivity of the results depend on the specificity of the primer pair and the probes used in replication. Standard amplification curves were examined for different species detection and the value of the coefficient of determination (R^2) was measured. The R^2 value is 0.984 for sheep, 0.973 for cattle, and 0.942 for chicken gallus species. Standard curves were plotted using the dilution series in the range of 10 ng to 100 femtograms. An analysis of the standard curves also showed that if the fluorescence values exceeding the threshold [CycleThreshold (Ct)] are greater than 35, they should be considered negative (when the DNA values in the sample are one copy or less).

In this study, the Real-time PCR method was used with the help of the Corbett Device (Qiagen, Germany) to determine cattle, sheep, and chicken species in the minced red meat samples. Primers and probes were prepared as a 10 picomol working solution based on the species in question. In this study, a commercial kit (Roche, Germany) was used and the master mix was prepared according to the manufacturer's instructions (Table 2):

Table 2. Master mix preparation steps

Reagent	Stock concentration	Reaction volume (25 µl)
Light cycler 480 probe master(2)	2X	10 µl
Primer Forward	10 Pm	1.5 µl
Primer Reverse	10 Pm	1.5 µl
Probe	10 Pm	0.5 µl
DNase & RNase-free water	-	1.5 µl
Final volume		1.5 µl

After preparing the master mix, 15 microliters of it were poured into 0.2 Real-time PCR microtubes and 5 microliters of DNase and RNase-free water were added to the negative control microtubule. DNA was then added to the microtubules (template) and 5 µl of DNA was extracted from the suspected samples. In the last step, chicken DNA was added as a positive control. The microtubes were centrifuged for 10-15 seconds and then transferred to Real-time PCR and the temperature command was applied (Table 3) and the results were reported in the Green channel.

Table 3. The execution of the temperature command

Real-time RT-PCR cycler conditions			
Step	**Time**	**Temperature**	**Cycle**
Pre-Incubation	10 min	95°C	1
Denaturation	10 Sec	95°C	45
Annealing and Elongation	60 Sec	60°C	

The sensitivity of this test was confirmed by various mixtures, including minced chicken mixed in beef, to detect the lowest amounts of chicken DNA. The minced beef percentages were 60, 50, 40, 30, 20, and 10% (by weight). Besides, DNA extracted from the beef, mutton, and chicken samples was tested for cross-reactions with each primer and probe, and DNA of other species was used as a negative control to detect any species. The reaction efficiency in most tests was close to 1, indicating that the efficiency of the primers in this method was close to 100%.

A positive amplification curve in the test indicates the presence of the relevant species in the samples. Given the abundance of mitochondria in cells, mitochondrial DNA copies (mtDNA) are high in each cell and species detection tests are more specific. Mitochondria extracted from cattle, sheep, and chicken by diluting samples in the DNA solvent buffer (Tris-EDTA Buffer) was prepared as a solution with concentrations of 10 ng to 100 femtograms including 10 ng, 1 ng, 100 picograms, 10 picograms, 1 picogram, and 100 femtograms per 100 µl of DNA solution.

Given that this study sought to investigate the presence or absence of minced red meat adulterations, all data were analyzed descriptively.

Results

In the histological analysis, of the 14 minced red meat samples with hematoxylin and eosin, Masson-trichrome, and PAS-alcian blue staining, 11 samples contained skeletal muscle and adipose tissue in addition to unauthorized tissues such as gizzard, koilin, and chicken skin, confirming the use of chicken gizzard and possibly the use of unauthorized tissues extracted from chicken in minced meat and showing the existence of adulteration in minced red meat (Figure 1).

Table 4 shows the results of the molecular analysis. In this study, 14 samples of minced meat were also tested by Real-time PCR.

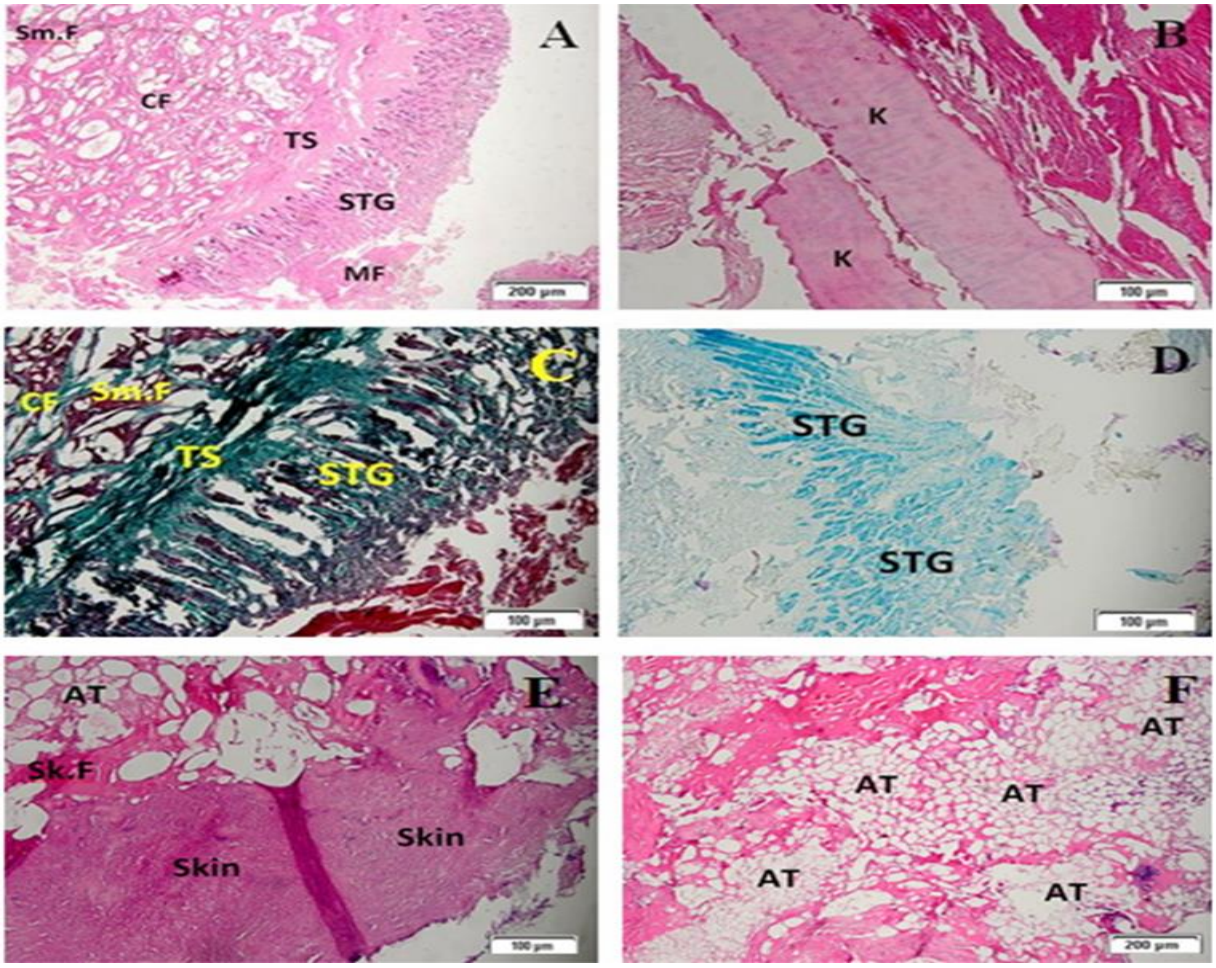

Figure 1. (A): Gingival tissue; straight tubular glands (STG); submucosa (TS); muscle fiber (MF), smooth muscle bundles (Sm.F); collagen fibers (CF). Hematoxylin and eosin staining
(B) Koilin in gingival tissue (K); Hematoxylin and eosin staining
(C): Gingival tissue. Straight tubular glands (STG), submucosa (TS), smooth muscle bundles (Sm.F); Collagen fibers (CF). Mason trichrome staining
(D): Positive reaction of gingival gland secretions with Pas-Alcuin Blue staining. Direct tubular glands (STG).
(E): Skin tissue in red minced meat (Skin), skeletal muscle bundles (Sk.F); Adipose tissue (AT), Hematoxylin, and eosin staining.
(F): Abundance of adipose tissue in minced red meat. Adipose tissue (AT). Hematoxylin and eosin staining.

Table 4. The results for 14 samples tested

Sample	Cattle	Sheep	Chicken
	Ct*	Ct	Ct
Positive control	11.89	22.94	30.13
Negative control	Negative	Negative	Negative
B1	15.04	Negative	19.97
B2	15.52	Negative	18.57
B3	14.38	Negative	16.72
B4	20.41	Negative	16.94
B5	14.12	Negative	24.94
B6	21.39	Negative	15.32
B7	Negative	Negative	12.48
B8	15.20	Negative	15.90
B9	26.47	Negative	Negative
B10	21.82	Negative	Negative
B11	23.35	32.22	22.79
B12	19.89	14.20	26.08
B13	19.79	Negative	24.53
B14	18.69	26.39	Negative

* Cycle Threshold.

As can be seen, 13 samples (93%) were beef, 3 samples (22%) were mutton, and 11 samples (79%) were chicken. Besides 11 samples (79%) contained chicken.

To check the accuracy of the results, it is necessary to check the limit of detection (LoD) and limit of quantitation (LoQ). To this end, the minimum LoD was calculated based on the method mentioned in previous studies (15, 16). The minimum limit of detection (LoD) in this test was 0.32 picograms for beef, 0.14 picograms for mutton, and 0.45 picograms for chicken, showing that the lowest limit of detection (LoD) in these samples ranges from 0.1 to 0.5 picograms. Real-time PCR used qualitatively in this test allows species detection in a wide range of DNA values. In this test, samples containing 0.0001% of beef and 0.001% of chicken and mutton can be detected. Therefore, the limit of detection (LoD) in the method used in this study was 0.0001% (10 fg/ml) for beef and 0.001% (100 fg/ml) for other animal species.

The quantitative data, amplification curves, and standard diagrams for the DNA of the tested animals (cattle, sheep, and chicken) are given in Tables 5 through 8 and Figures 2 through 4.

Table 5. The quantitative data for beef, mutton and chicken samples

Data	Beef	Sheep	Chicken
Standard Curve (1)	Conc= 10^(-0.306*CT + 10.688)	Conc= 10^(-0.248*CT + 10.830)	Conc= 10^(-0.218*CT + 10.734)
Standard Curve (2)	CT = -3.271*log(conc) + 34.960	CT = -4.034*log(conc) + 43.682	CT = -4.577*log(conc) +49.130
Reaction efficiency (*)	(* = 10^(-1/m) - 1) 1.02176	(* = 10^(-1/m) - 1) 0.76979	(* = 10^(-1/m) - 1) 0.65383
M	-3.27086	-4.03354	-4.57683
B	34.95976	43.68214	49.12985
R-Value	0.9869	0.99211	0.97107
R^2 Value	0.97398	0.98429	0.94297

Table 6. The qualitative data for beef detection

No.	Color	Name	Type	Ct	Given Conc. (Copies)	Calc. Conc. (Copies)
1		St6	Standard	29.78	10	38
2		St6	Standard	31.69	10	10
3		St5	Standard	29.89	100	35
4		St5	Standard	27.77	100	158
5		St4	Standard	25.52	1000	766
6		St4	Standard	26.14	1000	498
7		St3	Standard	23.11	10000	4198
8		St3	Standard	21.97	10000	9364
9		St2	Standard	18.62	100000	99036
10		St2	Standard	18.06	100000	146860
11		St1	Standard	14.46	1000000	1849222
12		St1	Standard	15.13	1000000	1158392
13		neg	NTC			

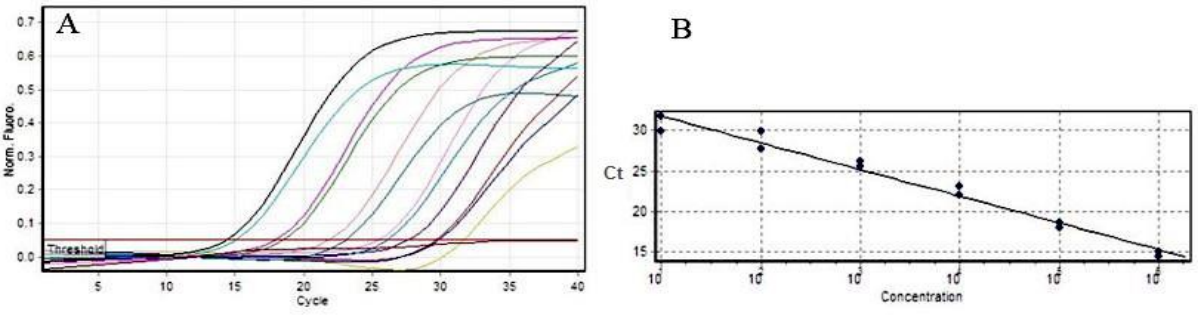

Figure 2. Amplification curves (A) and standard diagram (B) for beef detection

Table 7. The quantitative data for mutton detection

No.	Color	Name	Type	Ct	Given Conc. (Copies)	Calc. Conc. (Copies)
1		St1	Standard	24.10	100000	71719
2		St1	Standard	22.92	100000	140335
3		St2	Standard	27.05	10000	13264
4		St2	Standard	27.99	10000	7781
5		St3	Standard	31.02	1000	1379
6		St3	Standard	32.29	1000	668
7		S4	Standard	36.14	100	74
8		S4	Standard	35.01	100	141
9		A2	Unknown			
10		A2	Unknown			
11		B2	Unknown			
12		B2	Unknown			
13		neg	NTC			

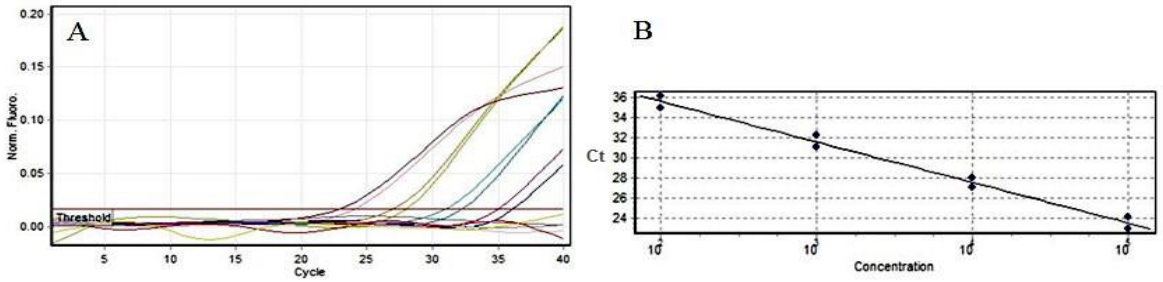

Figure 3. Amplification curves (A) and standard diagram (B) for mutton detection

Table 8. The quantitative data for chicken meat detection

No.	Color	Name	Type	Ct	Given. Conc. (Copies)	Calc. Conc. (Copies)
1		St6	Standard		10	
2		St6	Standard		10	
3		St5	Standard		100	
4		St5	Standard		100	
5		St4	Standard	35.80	1000	819
6		St4	Standard	33.33	1000	2830
7		St3	Standard	33.16	10000	3089
8		St3	Standard	31.03	10000	8995
9		St2	Standard	27.15	100000	63291
10		St2	Standard	25.26	100000	164482
11		St1	Standard	20.72	1000000	1615075
12		St1	Standard	21.83	1000000	923783
13		neg	NTC			

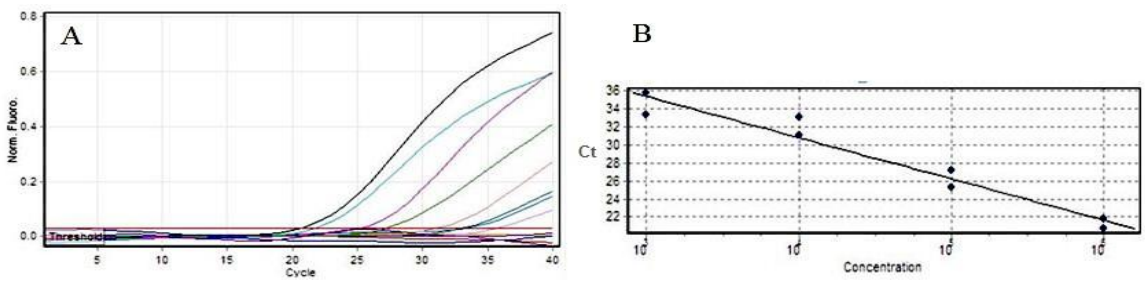

Figure 4. Amplification curves (A) and standard diagram (B) for chicken meat detection

Discussion

Chicken skin as one of the unauthorized tissues was detected in a number of the minced red meat samples. This tissue is characterized by the presence of two layers, the epidermis, which is thin and squamous, corresponding to the thin stratum corneum, and the dermis, which is thick and irregularly dense connective tissue and contains many capillary networks and arteries. Chicken skin is pink in hematoxylin-eosin staining, purple in PAS-alcian blue staining, and dark green in Masson-trichrome staining. These different stains helped significantly to identify the skin in the studied samples.

Sometimes, food standards are not observed when producing meat products and some people use unauthorized tissues in the production of these products, thus, histological tests, chemical control of meat products, or the use of DNA-based adulteration detection methods are useful and effective in reducing the risk of factors threatening consumers' health (17). Despite the improvements in the production levels and producers' compliance with standard requirements, there are still many cases of adulteration and substitution of undesirable materials during the processing of food products. Accordingly, microscopic analysis of food products is of particular importance for the detection of these cases. For example, according to the meat production standards, the use of animal tissues other than meat and fat is prohibited. The presence of gingival tissue in minced red meat is also considered adulteration (18).

Numerous studies have addressed food adulteration. For example, Sepehri et al. examined minced meat samples in Tehran using histological analysis and observed illegal tissues in minced meat (19). Barai et al. in Mumbai, India, compared different approaches to detect adulterations in meat products and confirmed the effectiveness of histological techniques (20). Adibmoradi et al. conducted a study in Tehran Province using histological tests and detected peritoneal adipose tissue, skin, kidney, and clear cartilage in meat products (21). Abbasi et al. reported the presence of breast, lymph nodes, and gingival tissues in meat product samples in Tehran Province (9).

Using histological analysis, Archer and Carey reported the presence of smooth muscle tissue as well as soy in meat products in Maryland, USA (22). Izadi et al. conducted a histological analysis in Yazd and detected chicken skin and adipose tissue in all tested samples in different percentages and showed that the histological techniques are effective in determining the quality and especially the quantity of chicken skin tissue mixtures (23). Sadeghi et al. confirmed the presence of unauthorized tissues in meat products produced in factories in Kermanshah Province, emphasized the increased concerns about the health of these products, and highlighted the exercise of more precise and complete control over the production of this group of food products (24).

In addition to the histological detection method, the application of Real-time PCR tests for reliable detection of the authenticity of meat in very complex mixtures is of particular importance. This technique can detect even very small amounts of different species in the product (7, 8). Various studies have explored this issue in recent years. As an example, Dooley et al. reported that Real-time PCR can detect the cytochrome b gene in beef, pork, mutton, chicken, and turkey in a mixture of raw compounds (25). Real-time PCR was also used by Hird et al. to determine deer meat and by Lopez-Andreo et al. to determine ostrich DNA and differentiate it from other species (26, 27). Chisholm et al. used DNA-based methods in commercial food products for the detection of horse and donkey meat (28). Druml et al. used real-time PCR to quantify deer meat, even in small quantities, in various other meat products. The 93.9% accuracy of the results and the high specificity of this technique confirmed its importance in detecting meat adulteration (29).

Rojas et al. identified pheasant, quail, pigeon, guinea fowl, and partridge meat using this technique. Besides, Fajardo et al. identified different components of mixed red deer, yellow deer, and antelope meat (30, 31). The effectiveness of this technique in investigating the possibility of mixing dog meat with chicken nuggets was confirmed in Malaysia and no mixing and therefore no adulteration was reported. This study reported a test accuracy of 99.7% (32). Various studies have shown that Real-time PCR has a specificity and sensitivity of over 93% (29, 30, 32). Histological techniques cannot easily detect the presence of chicken tissues in minced beef

or mutton, but Real-time PCR facilitates differentiation. The combined use of these two techniques was a unique feature of this study that has increased the accuracy of detecting food adulteration.

One of the limitations of this study was that it wanted to show the absence or presence of chicken meat or waste in minced beef, mutton, or a mixture of both, thus, the samples were analyzed qualitatively and as case reports, but the quantity was not assessed. Moreover, given the high cost of Real-time PCR tests and the qualitative focus of the study, the number of samples taken was limited to 14 samples. In most similar studies, only a laboratory method has been used. Similar studies have been performed using either molecular methods or histological methods. Since in the present study, both methods were used in a mixed design, increasing the number of samples could increase the time and cost of the study and this was one other limitation of this study.

Conclusion

Most of the minced red meat samples analyzed in this study contained unusual tissues, including gizzard tissue or chicken skin, whose unlabeled mixing was considered adulteration. Given the low price of chicken meat and the availability of its waste, chicken meat is mixed with red meat to reduce the price of minced meat, and this mixing is contrary to the defined hygienic and standard requirements and endangers the general health of the community. Molecular analysis in this study showed that small amounts of different animal DNA and adulteration in meat products can be easily detected through Real-time PCR.

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Conflict of interest

The authors stated no conflict of interest in this study.

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