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Chemical, Molecular, and Histological Techniques for Detection of Adulteration in Some Egyptian Meat Products

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THIS study aimed to detect the adulteration of some beef products (burger, sausage, luncheon) using chemical, molecular, and histological techniques. We used a total of 63 beef product samples (21 from each product) collected from Egyptian markets. Based on costs, these samples were categorized into class A (high price), class B (medium price), and class C (low price). Adulteration was detected using the chemical composition to estimate protein%, the high-resolution melting (HRM) approach of the real-time PCR (HRM-PCR) to detect species substitution, and the histological examination to detect the adulterated tissue. The chemical analysis showed significantly higher measured protein % in class A beef products than in Class B and C. Molecular detection of mitochondrial 16S rRNA using HRM-PCR revealed the presence of meat adulteration in class B and C by mixing beef meat with either meat of other animals (donkey, dog, and cat) which normally are not consumed or slaughtered in Egypt or meat of other closely related animal species such as sheep and goat. On the histological level, adulteration was also found as noticed by the presence of a large number of tissues other than meat including bone, cartilage, fat, connective tissue, and large blood vessels, especially in class B and C products. With these data, we could conclude that medium and low-price meat products had a high incidence of adulteration. We argue that this strategy for the detection of meat adulteration might be used to safeguard consumers against food fraud in developing countries like Egypt.

Keywords: Meat products, Adulteration, HRM, Histological examination, Protein.

Introduction

Products falling under the category of "processed meat" are those in which the meat has been combined with fat, water, and other ingredients. There is a vast variety of vitamins, minerals, and trace elements in meat products. High biological value, affordable pricing, pleasing taste, and simple preparation all contribute to the beef products (such as burger, sausage, and luncheon) popularity in Egypt as ready-to-eat meat products [1]. However, heavy consumption of these products has been linked to health problems such as heart disease, high blood sugar, tumors, liver, urinary, and lung disorders, thus they are generally avoided [2].

Common forms of meat adulteration that pose ethical, religious, and dietary issues include replacing high commercial-value meats with similar cheaper or unattractive species [3]. Fair trade is hindered by species replacement in meat products, which offends religious and ethical beliefs [4]. Undeclared meat species can cause foodborne or zoonotic infections and harm allergy sufferers [5]. Moreover, substituted species may be unsanitary and not pass meat

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inspection [6]. Meat adulteration was reported in some countries such as the UK and Ireland, where beef was swapped with horse meat, and China, where mutton was substituted with mouse flesh [5].

Analytical methods, including chromatography, mass spectrophotometry, imaging, and serology, can detect proteins, and metabolites of meat [7] but cannot determine species substitution. However, due to its higher specificity and sensitivity, DNA-based approaches have become increasingly popular for species substitution analysis in meat and meat products [8]. Using the *CO1*, *cyt b*, and 16S rRNA genes, previous research has shown that DNA sequencing may be used to detect beef product adulteration [9]. PCR with high-resolution melting (HRM) analysis can detect nucleotide sequence differences, such as SNPs and indels, allowing species genotyping [10]. To distinguish closely related species, HRM can evaluate DNA samples by comparing their dissociation curves, which are dependent on amplicon GC-content and sequence composition and length. This makes high-resolution melting analysis a valuable tool in food authentication [11]. Using HRM, previous studies quickly and inexpensively detected species substitution in meat [12].

Meat adulteration has a notable negative impact on health and the economy. For this reason, many developed countries set rules to prevent meat adulteration. However, there is a shortage of data about similar efforts in developing countries such as Egypt. Therefore, this study was conducted to detect the adulteration of some beef products (burger, sausage, luncheon) using chemical, molecular, and histological techniques.

Material and Methods

Preparation of meat samples

A total of 63 beef products (21 burgers, 21 sausages, and 21 luncheons) samples were purchased from different local markets in Kafr El-Sheikh city, Egypt. Based on costs, these samples were categorized into class A (high price, n = 7), class B (medium price, n = 7), and class C (low price, n = 7) for each meat product. About 20 g of each sample were gathered in sterile bags and stored in an ice tank until reaching the lab. For chemical analysis, samples were properly mashed in a blender (Moulinex, Paris, France). For HRM analysis, two 1 g sub-samples were thoroughly removed from each sample's interior portion using sterilized blades and gloves. After that, we placed the duplicates separately in 2 mL cryovials and kept them at -80 °C until DNA extraction.

Detection of crude protein percentage

To determine the protein percentage in all samples, the standard operating methods recommended by the Association of Analytical Chemists were followed [13]. The Kjeldahl method was utilized to determine the crude protein % by multiplying the total nitrogen by a fixed value of 6.25 [14].

Identification of species substitution by HRM-PCR

To determine the animal species, HRM-PCR analysis of the mitochondrial 16S rRNA was performed on DNA extracted from test samples (beef products) and standard (reference) samples (meat of cattle, sheep, goat, donkey, dog, and cat.) as previously described [12]. In brief, DNA samples were extracted from using genomic DNA extraction kit (Bioline, London, UK) per the manufacturer's instructions. The PCR-HRM was performed using Step OnePlus Real-Time PCR System (Applied Biosystem, USA) and specific 16S rRNA primers: forward 5'zGAGAAGACCCTRTGGARCTT3'and reverse 5'CGCTGTTATCCCTAGGGTA3' (Njaramba et al., 2021; Ouso et al., 2020). A 10-µl PCR mix was prepared by adding 5 µl of 2× Maxima SYBR Green/ ROX qPCR Master Mix (Thermo Scientific, # K0221, USA), 3 µl of DNA template (20 ng), and 1 µl of each primer (0.5 µM). In every run, there was also a negative control consisting of ddH2O, which was used in lieu of the DNA template. The thermal cycling conditions included: initial denaturation at 95°C for 15 minutes, 45 amplification cycles including DNA denaturation at 95°C for 20 seconds, annealing at 56°C for 20 seconds, extension at 72°C for 30 seconds, and one final extension step at 72°C for 5 minutes. To perform melting curve analysis, the temperature at the end of the last cycle was raised from 63 to 95 °C in 0.1 °C increments while monitoring the fluorescence every two seconds. The obtained fluorescence data was used to create graphs of melt rate [melting temperature (Tm) peaks] and normalized HRM using Step OnePlus software. Tm peaks analysis and normalization of test sample profiles to those of the reference species allowed for the differentiation of meat-source species.

Histological examination

Intact samples were fixed overnight in 10% buffered neutral formalin, dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin. The paraffin blocks were cut at 5 µm thick, stained with haematoxylin and eosin (H&E), and examined by a light microscope.

Statistical analysis

One-way analysis of variance was performed in GraphPad Prism (version 7) to calculate the difference in means between the groups. Means were compared using Tukey's test for statistically significant differences. Data were presented as mean + SEM, with a significance level of p value less than 0.05.

Results

Protein percentage in meat product

The protein percentage in three classes of commonly used Egyptian beef meat products (burger, sausage, luncheon) were analyzed, and a significant ($p \leq 0.05$) disparity was found between them (Table 1). We first compared the protein percentage as estimated by manufacturer (on-label) and as measured in our lab (measured) and found significantly lowered protein percentage in lab measured samples of class B burger, class C sausage, and class B and C luncheons as compared to their counterparts of on-label samples (Table 1). We then compared the measured protein percentage among the three classes of each meat product and found that class A exhibited a significantly higher ($p \leq 0.05$) protein percentage than class B and C in the three meat products. On the other hand, no significant difference was noticed between class B and C burger and sausage. However, the protein percentage in class B luncheon was significantly ($p \leq 0.05$) higher than that in class C.

TABLE 1. The protein percentage in beef meat products of the three classes.

Product	Class A		Class B		Class C	
	On label	Measured	On label	Measured	On label	Measured
Burger	14.00±0.58	13.33±0.88^{a}	9.50±0.29^{A}	7.17±0.44^{bB}	6.43±0.48	5.31±0.56^{b}
Sausage	12.83±1.17	11.93±1.21^{a}	5.65±0.22	5.43±0.23^{b}	5.30±0.38^{A}	3.67±0.24^{bB}
Luncheon	17.77±0.15	17.07±0.23^{a}	5.23±0.15^{A}	3.90±0.21^{bB}	3.67±0.33^{A}	2.10±0.21^{cB}

Data were presented as mean±SEM, n = 7/class/meat product. Means in the same row of the measured protein % had different lower-case superscript letters [a (highest value) – b (lowest value)] are significantly different at $p \leq 0.05$. Means in the same row between on label and measured protein % for each class had different upper-case superscript letters [A (highest value) – B (lowest value)] are significantly different at $p \leq 0.05$. On-label means as provided by the manufacturer; measured means as detected in our lab.

Molecular detection of meat products adulteration using HRM-PCR of 16S rRNA

The mitochondrial 16S rRNA gene with a fragment size of 200 bp was amplified from DNA reference samples of cattle, sheep, goat, donkey, dog, and cat meat using the HRM-PCR assay. The obtained results in form of melt curves, normalized melt curves and difference plot curve were considered as standard curves to detect any possible meat adulteration in beef meat products. Next, we extracted DNA from tested beef meat products (burger, sausage, and luncheon) purchased from commercially available local manufacturers. Table 2 summarizes the results of the HRM-PCR assay. As compared to standard (reference) samples, the HRM assay detected meat adulteration by mixing with meat of other animals which normally are not consumed or slaughter in Egypt. These species included donkey, dog, and cat. We also found adulteration by other closely related animal species such as sheep and goat. Beef burger was partially substituted with sheep meat (in class A), sheep and goat meat (in class B), and sheep, goat, and donkey (in class C). Beef sausage was partially substituted with sheep and goat meat (in class A and B), and sheep, goat, donkey, dog, and cat (in class C). Beef luncheon was partially substituted with sheep meat (in class B), and donkey, dog, and cat (in class C).

TABLE 2. Results of HRM-PCR show expressed species-specific 16S rRNA gene to detect meat adulteration of beef products by other species

Product	Beef	Sheep	Goat	Donkey	Dog	Cat
			Burger			
Class A	100% (7/7)	28.57% (2/7)	0% (0/7)	0% (0/7)	0% (0/7)	0% (0/7)
Class B	100% (7/7)	42.86% (3/7)	28.57% (2/7)	0% (0/7)	0% (0/7)	0% (0/7)
Class C	100% (7/7)	57.14% (4/7)	42.86% (3/7)	14.29% (1/7)	0% (0/7)	0% (0/7)
			Sausage			
Class A	100% (7/7)	42.86% (3/7)	14.29% (1/7)	0% (0/7)	0% (0/7)	0% (0/7)
Class B	100% (7/7)	42.86% (3/7)	28.57% (2/7)	0% (0/7)	0% (0/7)	0% (0/7)
Class C	100% (7/7)	71.43% (5/7)	57.14% (4/7)	28.57% (2/7)	14.29% (1/7)	28.57% (2/7)
			Luncheon			
Class A	100% (7/7)	0% (0/7)	0% (0/7)	0% (0/7)	0% (0/7)	0% (0/7)
Class B	100% (7/7)	28.57% (2/7)	0% (0/7)	0% (0/7)	0% (0/7)	0% (0/7)
Class C	100% (7/7)	0% (0/7)	0% (0/7)	42.86% (3/7)	28.57%	28.57%

The ratio shows the results of positive samples (expressed species-specific 16S rRNA gene) / total examined samples (n = 7).

HRM-PCR also showed a variation in melt temperatures (Tm) of 16S rRNA gene among various species with minor shift (Fig. 1). Tm ranged from 74.27 °C to 74.75 °C. Normalized melt curves were sufficient to identify the sequence variation with melting curve shapes (Fig. 2). As noticed in the provided figure, there were 6 different color clusters indicating the presence of 6 different animal species in all examined beef meat products. When data were analyzed by the difference plot curve, we obtained the same single curve shape but different colors representing animal species as shown in Fig. 3.

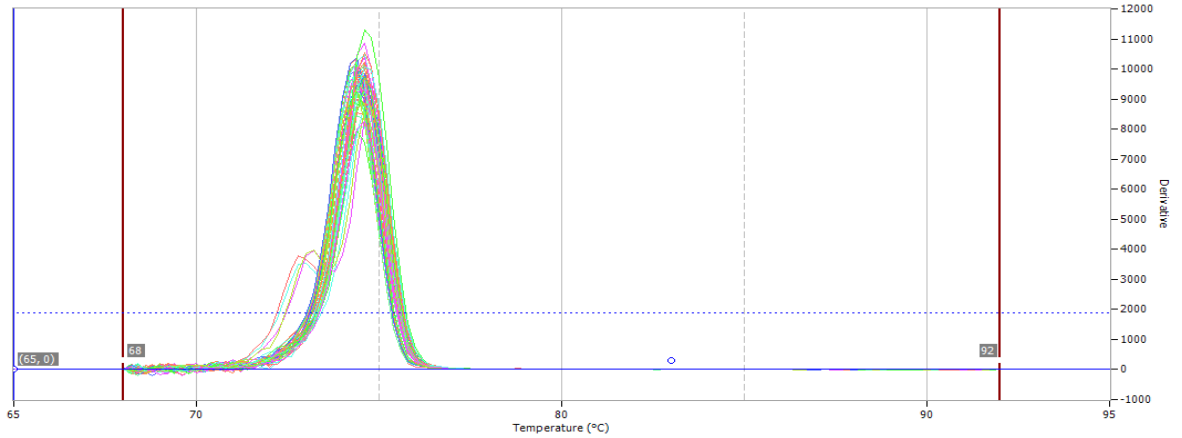

Fig.1. The melt temperatures (Tm) of 16S rRNA in all tested meat products indicate a minor shift.

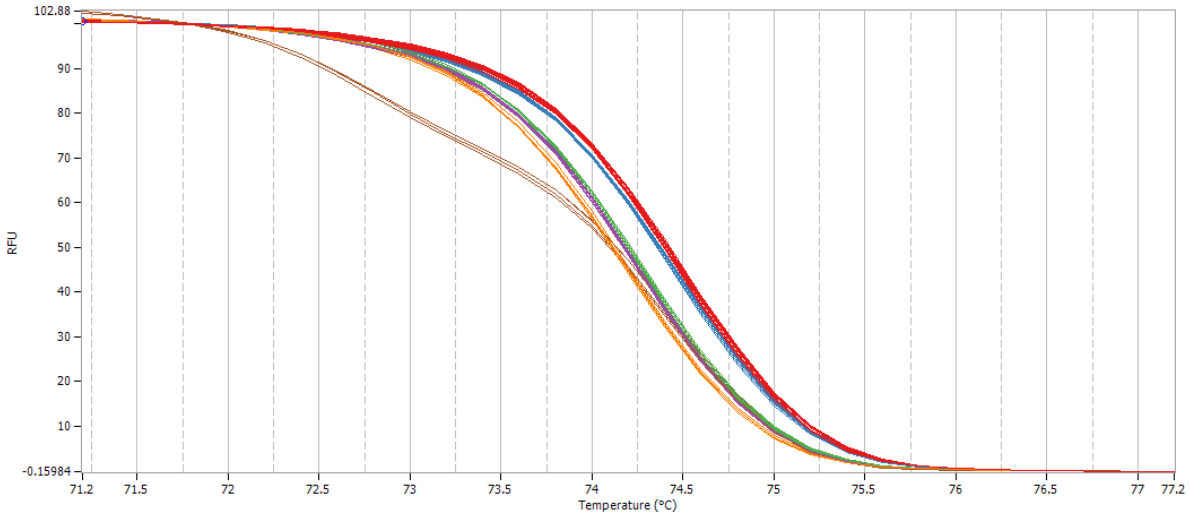

Fig.2. Normalized melt curves for all tested beef products. The colour key included red (cattle), blue (sheep), green (goat), purple (donkey), orange (dog), and brown (cat) as displayed by HRM-PCR software.

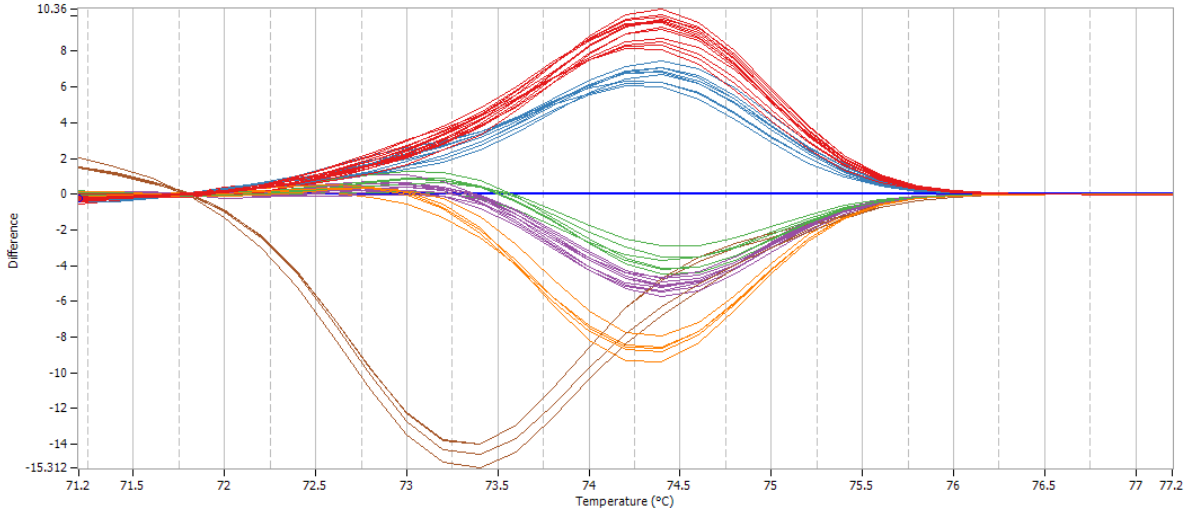

Fig.3. Difference plot curve for all tested beef products. The color key included red (cattle), blue (sheep), green (goat), purple (donkey), orange (dog), and brown (cat) as displayed by HRM-PCR software.

Histological examination of meat products

The histology of all H&E-stained sections were examined to detect tissue components in the beef meat products (Fig.4). In class A burger, the fibers of the skeletal muscle (white arrows) were numerous and well-arranged but with few bony spicules and central canal (black arrows), while in class B and C burger, the muscle fibers became fewer and disorganized (white arrows) with abundant number of bony spicules (black arrows and arrowheads). Comparing the three classes of burgers, class C had the lowest number of muscle fibers and the highest number of bony spicules.

In class A sausage, there was a large amount of organized skeletal muscle in cross section (white arrows), while in class B sausage the number of muscles fibers decreased (white arrow) and bony lamellae (black arrow) increased. In class C sausage, there were scantly muscle fibers and numerous large bony plates with characteristic histological structure (black arrow), surrounded by fibrous elements (white arrow). Again, the lowest number of muscle fibers and the highest number of bony spicules were observed in class C sausage. In class A luncheon, there were equally distributed and organized skeletal muscle fibers (white arrows) and a very few hyaline

cartilaginous elements (black arrows). In class B luncheon, there were a smaller number of skeletal muscle fibers (white arrow) surrounded by a very large amount of adipose tissue (black arrows) as compared to class A. On the other hand, class C luncheon had a large size artery with elastic lamina structure of its wall (white arrow), surrounded with scantly skeletal muscle fiber (black arrows).

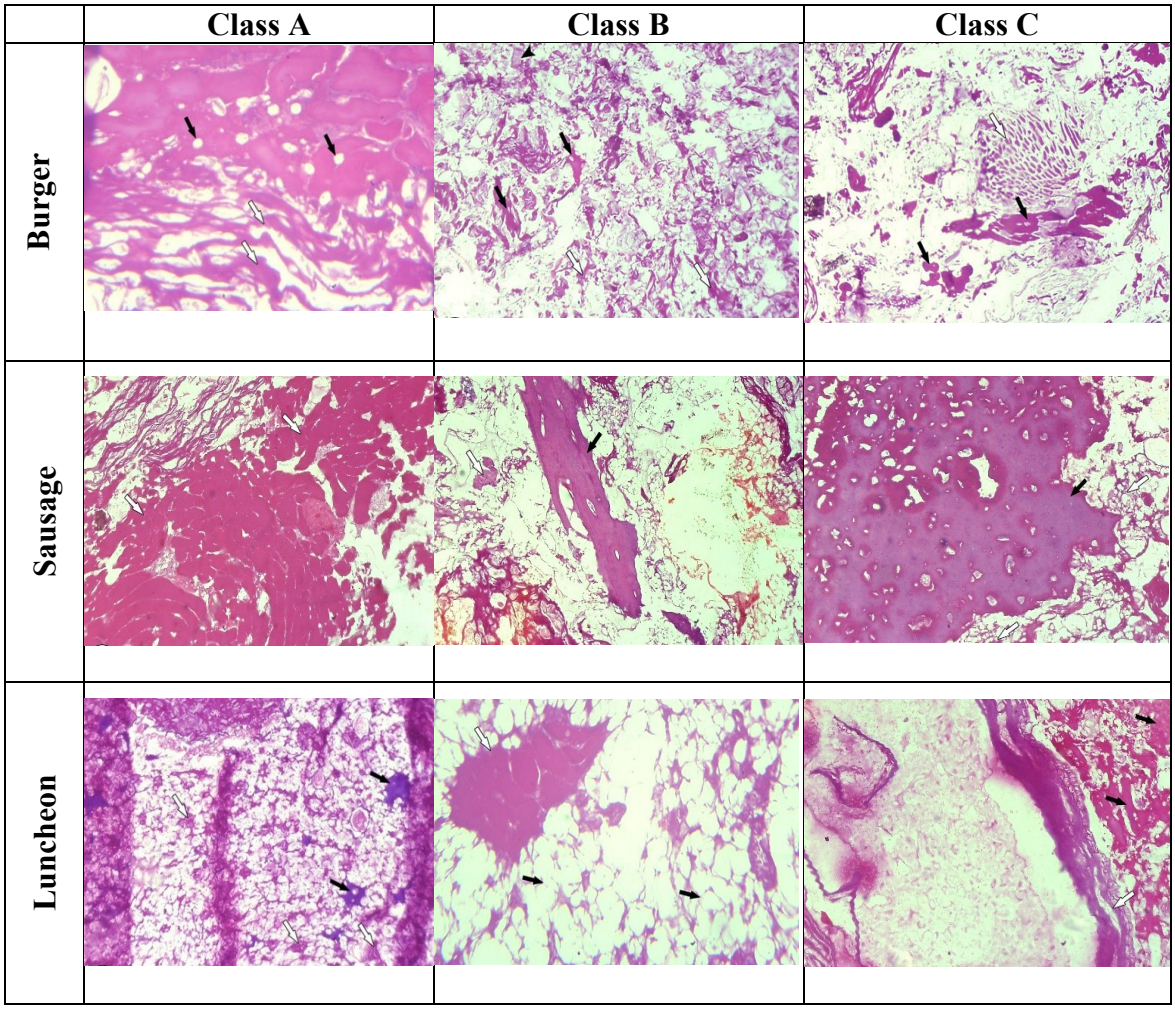

Fig.4. Detection of tissue components in the three classes of beef meat products (burger, sausage, luncheon) by histological examination of all H&E-stained sections. X = 40 in all images except class B luncheon where it equals 100. All arrows and arrowheads were explained in the main text.

Discussion

Meat's key component is protein, which not only provides customers with energy but also plays an important role in muscle growth [15]. Chemical analysis of the protein content in beef meat products revealed that class A (high price) burger, sausage, and luncheon had a significantly higher protein % than class B and C. Also, the on-label protein % in class A is nearly similar to the lab-measured protein %. This infers that class A Egyptian meat products could has superior nutritive value as compared to class B and C. Differences in protein content among the three classes can be attributable to the varied amounts of meat served or using cheaper non-meat products in place of actual meat in class B and C. Additionally, it worths to say that the high price of class A was found to have a correlation with its high protein content. This can also explain the wide gap between the prices of burger, sausage, and luncheon at different stalls in Egyptian markets. The protein content of Egyptian luncheon is specified to be roughly 16% per the Egyptian standard specification [16]. Subsequently, class A samples agreed with ESS, but Class B and C samples were all in disagreement with ESS. In agreement with our

results, a previous study reported a higher protein % in class A (16.10 ± 0.33%) Egyptian beef luncheon than in class C (3.50 ± 0.81%) [17], also obtained similar protein % in beef luncheon [18]. Moreover, Sabry et al., analyzed many types of traditional Egyptian beef luncheons and found their protein % ranged from 12.2 ± 0.04% to 15.74 ± 0.3% [19]. Essential amino acids could not be produced by the body and had to be obtained from meat and its products; hence, strict measures guaranteeing the availability of the allowed quantity of protein in Egyptian beef meat products could be imposed.

The HRM-PCR assay exhibited meat adulteration by mixing with meat of other animals which are not normally slaughtered in Egypt (donkey, dog, and cat) or with other closely related animal species (sheep and got). Based on HMR-PCR results, species substitution is widespread in class B and C meat products (burger, sausage, luncheon) sold at Egyptian local markets. Our general notice was the presence of the highest incidence of adulterated meats in low-price meat products. This type of meat adulteration by species substitutions could be intended in the low-price meat products (Class C) to increase meat volume using less expensive meat. However, it could also be unintendedly resulted from cross-contamination, such as contaminated knives or surfaces. The latter possibility is excluded in the present study as we used strict hygienic measures to avoid cross-contamination.

HRM-PCR also showed a very little variation (less than 0.5°C) in Tm of 16S rRNA gene among various species with a range of 74.27 °C - 74.75 °C regardless of whether they were raw, or cooked. Similar results obtained by Njaramba *et al.* (2021) who found Tm variation less than 1°C. This very little variation could be attributed to changes in salt content and DNA fragmentation which may affect PCR's effectiveness in beef samples exposed to different heat procedures [20]. Our ability to identify several species in mixed meat product samples using a 16S rRNA markers illustrates the power of HRM-PCR for this application. There have been an increasing number of reports of meat being adulterated with items derived from different species [21]. Consequently, this has led to an increased need for detection methods that are both economical and quick, the majority of these previous studies have used multiplex PCR to detect different vertebrate species in meat. While beneficial, multiplex PCR involves costly probes and post-PCR techniques (gel electrophoresis and sequencing), which prolongs analysis time, increases expense, and cross-contamination. In contrast to multiplex PCR, HRM-PCR makes it possible to conduct detection in real time while simultaneously reducing the number of processes and expenses required in the subsequent stages. Therefore, most recent studies and the present study have used HRM-PCR to accurately detect species substitutions in meat and its products [12]. Hence, it could be possible to use HRM-PCR to detect species substitution in commercially processed meat products (burger, sausage, luncheon), which are often contaminated with several undeclared meats. While DNA sequencing is the gold standard for identifying species, HRM-PCR analysis can aid in the finding of new, previously unknown species [12].

Adulteration of meat products using low-quality protein sources other than meat is a major source of concern for human consumption. To detect meat products adulteration by addition of other (not allowed) tissues, we histologically examined H&E-stained sections prepared from meat product samples. We found that only class A meat products had normal contents of muscle fibers with no or very little uncommon tissues such as bone and large blood vessels. However, class C had the lowest number of muscle fibers (less meat) and the highest number of bony spicules in addition to large blood vessels and fibrous connective tissues (fascia). This can explain the highest protein % in class A meat products and the lowest protein % in class C meat products. We also found an unusually large amount of adipose tissue in class B luncheon. Although fat plays a crucial role in the processed meat's organoleptic qualities and the goods' longevity, high consumption of meat products containing high fat content might be a major risk factor for obesity and being overweight [22].

Conclusion

Using chemical (protein %), molecular (HRM-PCR), and histological (H&E) approaches, this study shows that some medium and low prices Egyptian beef products (burger, sausage, luncheon) have been adulterated with low quality meat (less protein % and high levels of bones and large blood vessels) or meat from other species such as donkey, dog and cat which are not allowed to be slaughtered in Egypt. Therefore, it's important for consumers to read the labels of meat products and choose the best product based on its impact on health rather than the cost. Among the three approaches, HRM-PCR can efficiently and reliably detect species substitution in single- and mixed-species samples without DNA sequencing, making it the molecular tool of choice for food fraud surveillance in low-income countries such as Egypt. We suggest that our trios method of meat adulteration detection (chemical, molecular, and

histological) might be utilized to protect consumers from food fraud in poor nations like Egypt.

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الدراسات الكيمائية الجزيئية والهيستولوجيه للبحث عن الغش التجاري في بعض منتجات اللحوم في مصر

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[2] قسم الهستولوجي - كلية الطب البيطري جامعة كفرالشيخ- مصر.
[3] قسم التشريح- كلية الطب البيطري جامعة كفرالشيخ- مصر.

هدفت هذه الدراسة إلى الكشف عن غش بعض منتجات لحوم البقر (برجر، سجق، لانشون) باستخدام التقنيات الكيميائية والجزيئية والنسيجية. استخدمنا ما مجموعه 63 عينة من منتجات لحوم البقر (تمثل 21 عينة من كل منتج) تم جمعها من الأسواق المصرية كما تم تصنيفها إلى فئات بناء على السعر حيث تم تصنيفها الي ثلاث فئات مختلفة السعر المرتفع الفئة أ، السعر المتوسط الفئة ب، السعر المنخفض الفئة ج. البروتين، ونهج الانصهار عالي الدقة (HRM) في الوقت الحقيقي PCR (HRM-PCR)للكشف عن استبدال الأنواع، والفحص النسيجي للكشف عن الأنسجة المغشوشة. أظهر التحليل الكيمائي ارتفاع ملحوظ في نسبة البروتين في الفئة أ أكثر منها في الفئة ب و ج في كلا من المنتجات الأتية (البرجر، السجق، اللانشون). وباستخدام الطريقة الجزيئية للبحث عن الغش التجاري تبين وجود غش تجاري في الفئة ب و ج بواسطة خلط لحوم أنواع عينات أخري مثل الحمير والقطط والكلام مع لحوم البقر التي لا تصلح للاستخدام الأدمي والتي من المقرر ذبحها في مصر، كما تبين وجود لحوم لأنواع أخري من الحيوانات مثل الماعز والأغنام. وبالنسبة للطريقة الهستولوجيه لوحظ وجود أعداد ضخمة من أنسجة أخري غير اللحوم مثل: العظام، الغضاريف، الدهون، الأنسجة الضامة، الأوعية الدموية الضخمة خاصة في الفئة ب و ج. مع هذه البيانات، يمكننا أن نستنتج أن منتجات اللحوم ذات الأسعار المنخفضة والمتوسطة كانت ذات معدل مرتفع من الغش. نحن نري بأن هذه الاستراتيجية لاكتشاف غش اللحوم قد تستخدم لحماية المستهلكين من الاحتيال الغذائي في البلدان النامية مثل مصر.

الكلمات المفتاحية: منتجات اللحوم، الغش التجاري، إدارة الموارد البشرية، الفحص النسيجي، بروتين٪.