Molecular Identification of Normal and Adulterated Meat Samples of Local Markets and Restaurants in Kerbala Province

Al-Nasrawi Adil Ateyah Abd Ali[1], Ali Kadhim Aljarah[2], Haider Ali Muhammed[3]

[1]*Assist. Prof., Department of Environmental Health, College of Applied Medical Sciences ,University of Kerbala-Iraq.*

[2]*Assist. Prof., College of Sciences, University of Kerbala-Iraq.*

[3]*Assist. Prof., Department of Microbiology, College of Veterinary Medicine, University of Kerbala-Iraq.*

KEYWORDS
Mitochondrial Cytochrome b gene Species classification, Multiplex PCR, animal meats, and Staphylococcus aureus.

ABSTRACT
Background: Species specification of the meat is a highly important field of quality control management in meat consumption and industry. Meat forgery with undeclared sources has become a crucial trouble in many countries as well as in Iraq. It means that raw consumed meat or meat derivatives may include different meat genus; so, the components of meat are not regular like the known marker. The diet has sever effect on public health, religious factor, food safety principles, fair-trades and consumers desire.
Aim of the study: The current study was aimed to analysis the species of adulterated meat using molecular technique that targets Mitochondrial Cytochrome cyt b gene by Multiplex PCR for detection of meat species and authentication that provide many forensic and judicial applications for several populations throughout the world.
Materials and Methods: The following seventy (70) typical meat and meat derivatives were gathered from neighborhood super markets and eateries in the province of Kerbala: 25 cow, 10 buffalo, 25 sheep, and 10 goats. For meat samples, DNA extraction was done. Next, using species-specific primers of the Mitochondrial Cytochrome cyt b gene for cattle, sheep, goats, and buffalo, the collected DNA was amplified using the Multiplex PCR method. Additionally, the presence of Staphylococcus aureus, one of the microorganisms most commonly linked to food illness, was typically examined in all of the meat samples.
Results: Multiplex PCR technique resulting in species-specific products of the amplicon sizes for the DNA prepared samples with the lengths 472bp., 124bp., 585bp., and 330bp.
for the flesh of cow, buffalo, sheep, and goats, in that order. In addition to this analysis technique indicated that8 (44.4) % of examined cattle meat samples were mixed with buffalo meat and10 (55.6) % of examined sheep meat samples were mixed with goat meat. As well as, most of examined (normal and mixed) meat samples were contaminated with Staphylococcus aureus bacteria.
Discussion: It has been demonstrated that the highly conserved Mitochondrial Cytochrome cyt b gene primers, when used in the multiplex PCR methodology, provide a sensitive, straightforward, adaptable, and dependable method for the investigation of meat samples and meat products.
Conclusions: The current study's conclusions state that the “mitochondrial cytochrome cyt b gene” is a useful marker for differentiating between normal and adulterated meat. It also states that Staphylococcus aureus was found in 16/52 (30.76%) of the normal meat samples and 12/18 (66.7%) of the adulterated meat samples, with a significant importance of P=0.046.

1. Introduction

Adulteration is a process in which there is a exchange of an expensive meat with an inexpensive one, it is a severe problem for consumers and researchers and has provoked to discover a suitable strategy for ideal meat authenticity (Jain et al., 2007). Froudulation in the meat foods like sausage and hamburger and others has been a widespread concern in restaurants and super markets especially in developing countries as well as in Iraq. Actually, meat derivatives adulteration means addition or replacement of animal protein derivatives of inexpensive prices or proteins of plants such as soybean with fake weights of exact components (Dooley et al., 2004).

Classification of the origin or species of meat and meat products is necessary and extremely significant for peoples and health domain for numerous considerations like specific food allergies, economic and legal factors, religious affairs, and medical aspects (Rodriguez et al., 2004; Arslan et al., 2006; Mane et al., 2009). The consumers require very clear and accurate information about their diet and the foods to make sure in choosing one food product over another. Nowadays, numerous meat and meat derivatives may include various meat species in diverse quantities mixed mutually, so, cannot be detected by the naked eye or by feeding (Zarringhabaie et al., 2011).

The law of Islamic religious is prevent the Muslims from ingestion of pig, dog meat and their product, it is (Haram) for Muslims, as well as donkey and horse meat which is undesirable (Makrooh) for Muslims, thus existence of pork and horse or donkey meats or their products is improper for the Muslims nations, although the adulteration is by chance and in slight level. As well as, these animal species could be obtained and lacking any presented costs and consequently there is a large probability of including them in halal diets (Rahman et al., 2014; Ali et al., 2020). In this admiration there were several prior published reports indicated adulteration in normal meat or processed meat products that disseminated in many world countries particularly in Islamic nations like Iran (Ghorban Elyasi Zarringhabaie et al., 2011; Mehdizadeh et al., 2014; Al-taghlubee et al., 2019), Turkey (Osman and Hüsnü, 2015) and Iraq (Abdul-Hassan and Tauma, 2014; Hassan et al., 2019).

Various analytical methods were depended for determining adulteration of meat and its derivatives, for example the Spectroscopy analysis (Ding and Xu., 1999) Chromatographic Assay (Derya ARAÇ* et al., 2022), Immunochromatographic (IC) assay (Depamede, 2011), ELISA technique (Asensio et al., 2018), Histological controls (Eser and Nesrin, 2018) and Raw Meat FlowThroughTM Test (RMFT) (Ali et al., 2020).

Furthermore, proteomic advances depending upon classification of diverse biomarkers of peptide has been urbanized as well as engaged to provide information on the special components of diet products (Farag et al., 2015; Elena et al., 2020). Although all the protein employing techniques are reliable for species identification, but they have diverse cons and pros according to the employer's objectives and requests, the pros of these techniques they are not applicable for using regularly because they are not specific enough (Esteki et al., 2019).

Beside, DNA based techniques like (PCR) have been widely used for species determination and quantification analysis of animal species for meat and meat products, milk and dairy products because they have the stability, sensitivity and specificity such as Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) (Arslan et al., 2005), Restriction fragment length polymorphism (-RFLP) (SAFIYYAH SHAHIMI et al., 2018), PCR-nucleotide sequencing (Yong Hyun Park et al., 2013), Micro Drop Digital PCR (Chen Chen et al., 2020), QPCR(Aina et al., 2019), TRIPLEX PCR (Muhammad, 2022) and End-point of Multiplex PCR (Sangthong et al., 2021).

Furthermore, mitochondrial DNA (mt DNA), such as mitochondrial Cytochrom b genes, has been established as potent indicator tools in species determination by DNA investigation techniques. DNA-based analysis has used various genes or fragments of genes to investigate the origin of foods or animals (Andrejevic et al., 2019). According to Prusak et al. (2004), it is also necessary for various uses in legal medicine, forensic investigations, and molecular developmental studies.

Small DNA fragments of the MT-cyt b gene, which are notably rare in low DNA quantity or degraded materials, can be effectively used to recognize species (30). In order for the identification test to remain highly sensitive and specific for the species concerning the developed primers, it is necessary to ensure the specificity of primer from other animal primers when developing species specific primers of Mitochondrial Cytochrom b genes for species identification (Kim et al., 2020; Mayada et al., 2020).

Most of Iraqi people have an importance in selection of their diet and they prefer specific types of meat and meat products for daily consumption more than other meats, for example; they favor local cattle meat and meat products more than cattle and buffalo meat particularly that imported from India or other foreign countries, as well as, they prefer Iraqi sheep meat more than goat meat or other types of meat from foreign sources, this issue has significant importance and impact for meat consumers of Iraqi people through selection the types of diets, public health, religious factors, economic aspect.

The most pathogenic bacteria which associated with contamination of meat and meat products

include, Escherichia coli, Salmonella spp., Staphylococcus aureus, Clostridium perfringens, Listeria monocytogenes and Campylobacter jejuni (Pal et al., 2018). Staphylococcus aureus is a Gram-positive cocci and it is a part of the family of Micrococcaceae, which originates a large number of diseases and constitutes one of the etiological agents that cause various pathologies in humans such as skin and soft tissue infections and bacteremia (Kahl et al., 2016). it is traditionally considered a pathogen because it is related to nosocomial infections; the ability to produce food poisoning, in this sense Staphylococcus aureus can multiply rapidly in food and food products, generate a large number of bacterial colonies without there being evidence of food spoilage (Abdel-Atty et al., 2020).

1.1 Aims of the study: the most important objectives of the present study were employing of the DNA based molecular technique (Multiplex PCR method with species-specific primers of Mitochondria Cytochrom b genes for simultaneous determination of animal species of some meat and meat products samples, and detection the percentage of contaminated meat samples with Staphylococcus aureus bacteria.

2. Methodology

Materials :

Reagents & Solutions:

-QIAamp DNA Minikit (Qiagen, Germany, GmbH)

-Proteinase-K

- ATL buffer

- AL buffer

- Ethanol solution (100% concentration)

- Elution buffer

- Emerald Amp Max PCR Master Mix (Takara, Japan)

- Agarose powder (Appli Chem, Germany, GmbH)

- 1x TBE buffer

- Ethidium bromide

- Tellurite Glycine Agar Tellurite Solution 1% medium

Materials and Equipments:

- Nanodrop spectrophotometer (Thermo Scientific, Willington, DE, USA) (Model ND1000)

- Thermal cycler device (Clever scientific)

- Electrophoresis device

- Transilluminator with UV source

- Gel documentation system (Alpha Innotech, Biometra)

Samples:

- 70 meat samples (10 normal cattle meat, 15 cattle meat products (5 sausage, 5 hamburger, and 5 Lunshon meat), 10 buffalo meat samples, 25 normal sheep, and 10 normal goat meat samples) as, well, as

- Positive control samples (fresh cattle, buffalo, sheep, and goat meat samples).

Samples collection: Seventy (70) meat samples include (10 normal cattle meat and 15 cattle meat products: 5 sausage, 5 hamburger and 5Lunshonmeat and 10 buffalo meat samples, 25 normal sheep

and 10 normal goat meat samples were utilized in this study were purchased and randomly collected from traditional super markets and restaurants of various regions in Kerbala province, Iraq during January 2022. All of the meat samples were refrigerated, then, transported to the clinical molecular laboratory with refrigerated conditions, meat samples were instantaneously processed or frozen preserved at a temperature(-20ºC) for the next examination step. moreover, two samples of fresh cattle, buffalo, sheep and goat meat samples were afford to be employed as a positive control.

Methods

The following procedures were followed to extract DNA from the meat samples and its derivatives using the "QIAamp DNA Mini kit (Qiagen, Germany, GmbH)": Twenty-five (25) milligrams of every beef sample were treated for an overnight period at 56ºC with 180 µl of ATL buffer and 20 µl of proteinase K. Following a 10-minute incubation period at 72ºC, 200 µl of AL buffer and 200 µl of a 100% concentration ethanol solution were added to the lysate. After that, the lysate was quickly moved to a silica column (a column made with a DNA extraction kit), and it was centrifuged. The sample was then cleaned and centrifuged in accordance with the manufacturer's instructions. The DNA sample was eluted using 100 µl of the elution buffer included in the kit. According to Shayan et al. (2018), extracted DNA was divided into tiny amounts (5 µl), tagged, and kept at -20 °C until molecular analysis.

Using a Nanodrop spectrophotometer (Thermo Scientific, Willington, DE, USA) (Model ND1000), the absorbance at OD260 nm was calculated to determine the concentration and purity of the DNA. As an alternative, the ratio of absorbance at OD260 to OD280 nm was used to calculate the purity of the DNA.

After extraction, DNA samples were amplified with species specific oligonucleotide primers, these primers were obtained from Metabion company in (Germany),they are scheduled in Table1, these primers were species-specific for cattle, buffalo, sheep and goat according to preceding similar comparative study (Ghorban Elyasi Zarringhabaie et al., 2011).

Oligonucleotide primers: species-specific primers of distinguishable fragments of the genome of mitochondria were selected for DNA amplification. The sequences of primers have been targeted the mt DNA of Cytochrome b (cytb) gene of diverse species. Each Partial-length of mitochondrial cyt b gene was amplified with the specific primer pairs. Reverse primer was designed by prior researchers, used as universal Reverse primer (R) and five different Forward primers (F) of mt DNA of Cytochrome b (cytb) gene were utilized for amplifying species-specific segments for the meat samples and their derivatives for the domestic animals: cattle, buffalo sheep and goat (Ghorban Elyasi Zarringhabaie et al., 2011). as demonstrated in (Table 1).

Table 1: Oligonucleotide primers set of “Mitochondrial cytochrome b (cytb) gene” of Animal Species with the Sequence 5'-3' and amplified products and Reference.

Specific species	Primer Sequence 5'-3'	Amplified product size bp.	Reference
Common-Reverse Primer	R-TGTCCTCCAATTCATGTGAGTGT	-	Ghorban E. Z. *et al*., 2011
Cattle Forward Primer	F-TCCTTCCATTTATCATCATAGCAA	472	Ghorban E. Z. *et al*., 2011
Buffalo Forward Primer	F-TCCTCATTCTCATGCCCCTG	124	Ghorban E. Z. *et al*., 2011
Sheep Forward Primer	F-TACCAACCTCCTTTCAGCAATT	585	Ghorban E. Z. *et al*., 2011
Goat Forward Primer	F-CGCCATGCTACTAATTCTTGTT	330	Ghorban E. Z. *et al*., 2011

Table 2: PCR cycling conditions

Primary denaturation	**Amplification (34 cycles)**			**Final Extension**	
	Secondary denaturation	**Annealing**	**Extension**		
94°C for 3 min.	94°C for 45 sec.	62°C for 1 min.	72°C for 45 sec.	72°C for	10 min.

Multiplex PCR

Primer-based DNA amplification was carried out in a 25 µl reaction Eppendorf tube. To achieve the required reaction volume of 25 µl, it included 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each F and R primer at a concentration of 20 pmol, 5 µl of DNA template, and 3.5 µl of deionizer water. Using the PCR cycling conditions given in Table 2, the PCR reaction was conducted in a Clever Scientific heat cycler equipment.

Analysis of the PCR products: The gel electrophoresis method was used to separate the PCR amplification products. An agarose gel was made by dissolving 1.5 g of agarose powder (Appli. Chem. Germany, GmbH) in 100 ml of 1x TBE buffer, the suspension was heated at 90 °C in water bath until it becomes completely soluble and clear, the agarose was cooled to 50 °C, and 2 ul.of a concentration (0.5 µg/ml) After adding ethidium bromide to the agarose gel, it was poured into the gel tray and allowed to solidify at room temperature to form agarose gel, then, 5 ul. of each of DNA product samples were loaded with loading dye in the agarose gel wells with Gene ruler of 100 bp. DNA ladder from (Fermentas, company in Germany) was utilized and the electrophoresis process was performed in electrophoreses device using 7V/cm. gradients at room temperature for 1 hour. Following the completion of electrophoresis, the DNA fragments were visualized by Transilluminator with UV, then, photographed by gel documentation system of (Alpha Inno tech, Biometra) (Hamouda Ahlam et al., 2020).

For the sake of determination of meat contamination, 100 - 300 gm. of the four types of meat were enrolled cattle, buffalo, sheep, and goat. In each sampling unit, duplicate samples were taken, meat swabs were performed from all of the samples, then streaked on Tellurite Glycine Agar Tellurite Solution 1% medium which was utilized to identify Staphylococcus aureus according to manufacture company, the cultures with presumptive growth of Staphylococcus aureus were confirmed with routine microbiological methods. The reading unit (CFU/g.) of the bacterial growth of samples was carried out based on Food and agriculture organization CXC 58-2005 Codex Alimentariun, which establishes a range of 100-1000 CFU/g as acceptable quality criteria for meat and meat products samples. Colony counts were performed and expressed in CFU/g. The samples in which no growth was observed were reported as <100 CFU/g.(Siriken, 2004).

2.3 Statistical Analysis

Social science software Version 25 of statistical package was utilized for the comparison of the percentages of different samples of adulterated (mixed) meat by Fischer's Exact in addition to, Chi-square tests. Statistical analysis at P values less than, 0.05 were statistical significant in consideration.:

3. Results and discussion

The meat samples from cattle, buffalo, sheep, and goats in the current investigation had extracted DNA concentrations of 320, 224, 475, and 217 µg/ml, respectively. The proportion of OD260/OD280, as evaluated with a spectrophotometer, was between 1.8 and 2.2.

As shown in Table No. 3, the total number and percentage of adulterated (mixed) meat samples in the study was 18/70 (25.71)% with a significant importance of P= 0.033. These samples were divided into 8/18 (44.4%) cattle meat samples that were mixed with buffalo meat and 10/18 (55.6%) sheep

meat samples that were mixed with goat meat. The percentages of each group had a significant importance of P= 0.024 and 0.047, respectively, and there was no difference or normalcy in the buffalo (10/52 (19.23) % and the goat (10/52 (19.23) % meat samples.

Moreover, amplification of specific fragments of mitochondrial Cytochrome b gene with specific primers utilizing Multiplex PCR and DNA sizing method with agarose gel electrophoresis was displayed the amlicon of DNA products with the length of (472bp., 124 bp.) for (Bos indicus and Bos taurus) (cattle) and Bubalus bubalis (buffalo) for the pure and mixed meat samples respectively as exhibited in figure (no.1, 2) in addition to, DNA products with the length of (585bp., 330 bp.) for Ovis aries (sheep) and Capra hircus (goat) meat samples respectively as exhibited in figure (no. 3).

Concerning the detection of bacteriological contamination of animal meat samples, the current study demonstrated that 16 (30.76%) of total 52 normal meat and meat products samples were contaminated with Staphylococcus aureus, counts > 100 CFU/g., were distributed as the following: 5/17 (29.41%), 2/10 (20%), 5/15 (33.3%), and 4/10 (40%) both in cattle, buffalo. sheep, and goat meat samples respectively, with the significant importance (P=0.046) table 4, as well as, 12 (66.7%) out of total 18 adulterated or (mixed) animal meat samples were contaminated with Staphylococcus aureus, counts > 100 CFU/g., were distributed as the following: 7/8(87.5%), and 10/10 (100%) both in cattle and sheep meat samples with the significant importance(P=0.046) table 4, that appeared according to macroscopic inspection analysis of the bacterial culture characteristics of Staphylococcus aureus on Tellurite Glycine Agar medium as displayed in (figure no. 4).

Discussion:

For religious, legal, economic, and public health reasons, meat counterfeiting is a major global issue that is also outside of federal and state regulatory boundaries (Mafra et al., 2008; Abbas et al., 2018). Moreover, the Food and Drug Administration ("FDA") in the United States noted that food adulteration frequently indicated a violation of safety or health regulations. A few years ago, the prevalence of fake meat and its byproducts increased dramatically, especially in emerging and undeveloped nations. Customers are becoming more conscious of food safety and labeling statements as a result of food fraud and adulterations (Tibola et al., 2018).

Many investigators applied various quantification and identification assays in proteomic analysis like immunological and electrophoretic methods for identification of adulteration of meat, milk and other foods, these procedures were not enough sensitive to heat-treated meat and milk because of protein denaturation during processing, as well as, these procedures are labor-intensive, time-consuming and relatively expensive (Poonia et al., 2016). Other researchers employed DNA based PCR amplification for identification and typing of fraud meat and processed foods because DNA considered as a high stable molecule compared to proteins which allows analysis for heat treated and processed food. The troubles which may present during the classification of animal species by meat or food products samples were largely overcome by the using of DNA based techniques as ensured by all of the previous investigators, The PCR technique takes a special consideration since, it is specific, sensitive, relatively fast analysis procedure (Tanabe et al., 2007; Yosef et al., 2014).

The results of the current research have revealed relative similarity with other prior studies that referred to presence of adulteration in meat and meat derivative samples with relatively differences or ratios according to regions and DNA samples during the determination of animal species from meat, other processed food using molecular techniques (Dooley et al., 2004; Arslan et al., 2006; Jain et al., 2007; Zarringhabaie et al., 2011).

The target DNA which utilized for classification of meat species was mitochondrial Cytochrome b (cyt b) gene, it was earlier biomarker that depended to distinguish the misrepresented animal meat species is compatible with several previous studies (Al-taghlubee et al., 2019; Ali et al., 2020; Mayada et al., 2020; Sangthong et al., 2021). Beside, the result in (Figure-3) of Multiplex PCR displayed the species specific amplified PCR products for mitochondrial Cytochrome b gene with the

sizes of 585 bp. and 330 bp. for sheep and goat respectively for the pure and mixed meat of those species.

Both nuclear and mitochondrial genes were extensively aimed for classifying of animal meat and meat derivative species. The (mtDNA) was established as valuable markers than nuclear genome in species recognition and verification because the (mtDNA) is maternally inherited and it has changeable regions originated in numerous copies per individual cell which make possible PCR amplification, enhance sensitivity of technique and getting acceptable result even if the severely fragmented DNA or DNA damaged during powerful conditions of food processing as improved by preceding studies(Muangkram et al., 2018; Andrejevic et al., 2019; Mayada et al., 2020). These properties provide the largest chance for mitochondrial DNA to be reliable and highly efficient markers than nuclear ones for recognizing the origin of meat species in the procedures of investigations of processed meat species confirmation in new, heat treated meats.

Similar studies have demonstrated that the short fragment sequence of the mitochondrial cytochrome b gene, which serves as a universal DNA identity for animal species, is maintained. The current study's findings demonstrated that, as suggested by (Yacoub et al., 2013; Hadj-Henni et al., 2015 ; Mayada et al., 2020), the mitochondrial cytochrome b (Cyt b) gene was a viable marker for animal species confirmation. It is a highly successful method for identifying both domestic and wild animal types.

In the present study, specific primers of mitochondrial cytochrome b gene was applied in Multiplex PCR technique with the using of a universal Reverse primer and precise Forward primers for cattle, buffalo, sheep, and goat. Multiplex PCR permits the concurrent magnification of several DNA targets in one reaction, It is gradually fitting both with scientific investigations and conventional analyzing laboratories, it is agree with the previous similar studies who employed one conserved primer with various other primers in simultaneous multiplex PCR methods (Ghorban Elyasi Zarringhabaie et al., 2011; Abdul-Hassan and Tauma, 2014; Mayada et al., 2020).

In addition to, employing of molecular method (Multiplex PCR technique) in the present analysis with species specific primers was similar to numerous comparable survey of previous studies that assumed several primers together for simultaneous amplifying multi DNA fragments, all of the investigators ensured that Multiplex PCR technique is sensitive, efficient, applicable, reliable, inexpensive, and a hopeful technique which enhance the speed of investigation for the instant and obvious detection of numerous meat species (Tobe and Linacre, 2008; Lanping et al., 2019; Al-taghlubee et al., 2019; Sangthong et al., 2021; Muhammad Sulyman Saleem, 2022).

It is commonly known that a wide range of microorganisms may either directly or indirectly contaminate meat and meat products by contact with blood, the contents of the gastrointestinal tract, feet, hide or skin, water, knives, equipment used in slaughterhouse vehicles, and staff members. The most frequent bacteria found in meat include E. Coli, Staphylococcus aureus, Listeria monocytogenes, Salmonella, Aeromonas species, Arobacter species, Bacillus cereus, Campylobacter species, Clostridium botulinum, and Helicobacter species. Before animal meat is consumed by customers, veterinary inspectors should reject any infected meat that contains Bacillus anthracis, Mycobacterium TB, and Brucella abortus (Javed, 2016; AttAullAh Bughti et al., 2017).

On the other hand, Tellurite Glycine Agar medium was exhibited the characteristic properties of the considered presumptive growth of Staphylococcus aureus and appeared as black colonies were observed due to reduction of Staphylococcus aureus of tellurite to tellurium when it was added to Tellurite Glycine Agar medium and double halos around the colonies due to the presence of the phenomenon of lipolysis and proteolysis produced by the action of lipases and proteases enzymes that present in developed bacterial colonies (figure no. 4) as reported by prior study by (Ebovitz et al., 1955).

4. Conclusion and future scope

Identification of adulterations in normal meat and meat derivatives is crucial for many purposes including the protection of meat consumers, avoidance of miss labeling legislation and anticipation of unfair competition. For these reasons, simple, sensitive, precise and applicable

Multiplex PCR method was used for routine application in detection of meat and meat derivatives origin. Mitochondrial genome with the using of Mitochondrial Cytochrome b (Cyt b) gene was proved to be suitable and recommended marker in legal and forensic classification of foods. In light of the results of this study, 18/70 (25.71) % of meat samples were mixed with the meat of other species, distributed as the following: 818/ (44.4) %of cattle meat samples were mixed with buffalo meat (P=0.024) and 10/18 (55.6) % of sheep meat samples were mixed with goat meat (P=0.047). Indeed, 16/52(30.76%) of normal meat and 12/18 (66.7%) of adulterated meat samples were contaminated with Staphylococcus aureus, with the significant importance P=0.046. Finally, we submit the recommendation for increasing the number of meat and meat products samples in larger areas and sources of meat, as well as, investigation of the types of undesirable meat in Iraqi population such as haram (pork meat and meat derivatives) and makrooh like donkey, and horse meat with the employing the molecular technique and species specific gene that depended in the current study.

Declarations

We attest that this work is unique, unpublished, and not presently being considered for publication anywhere else. The "University of Kerbala" representative of my institute is completely aware of this contribution, and all of the writers concur. This work does not require ethical approval. There are no disclosed conflicts of interest.

Acknowledgments

The technicians and faculty members of the University of Karbala’s Faculty of Veterinary Medicine College, Microbiology Department, and Molecular Biology Laboratory are all acknowledged by the authors of this work for their assistance during the research process

Reference

[1] Jain S., Brahmbhait M.N., Rank D.N., Joshi C.G., and Solanki J.V. (2007). Use of *cytochrome b* gene variability in detecting meat species by multiplex PCR assay. *Indian Journal of Animal Sciences*. 77: 880-881.

[2] Dooley JJ, Paine KE, Garrett SD, Brown HM. (2004) Detection of meat species using TaqMan real-time PCR assays. *Meat Sci*. 68 (3) p. 431-8.

[3] Rodríguez M. A., García T., González I., Asensio L., Hernandez P.E., and Martin R. (2004). PCR identification of beef, sheep, goat, and pork in raw and heat-treated meat mixtures. *Journal of Food Protection.* 67: 172-177.

[4] Arslan A., Ilhak O.I., and Calicioglu M. (2006). Effect of method of cooking on identification of heat processed beef using polymerase chain reaction (PCR) technique. *Meat Sci*.72: 326-330.

[5] Mane B.G., Mendiratta S.K., and Tiwari A.K. (2009). Polymerase chain reaction assay for identification of chicken in meat and meat products. *Food Chem*. 116: 806–810.

[6] Zarringhabaie, G. E., N. Pirany and A. Javanmard, (2011). Molecular traceability of the species origin of meats using multiplex PCR. Afr. *J. Biotechnol.*,10: 16461-16465.

[7] Rahman, M.M., M.E. Ali, S.B. A. Hamid, S. Mustafa and U. Hashim *et al*., (2014). Polymerase chain reaction assay targeting cytochrome b gene for the detection of dog meat adulteration in meatball formulation. *Meat Sci.,* 97: 404-409.

[8] Ali M. Ahmed1, Takwa H. Ismail 2, Reham R. Abouelmaatti 3, Rehab E.M. Gaafar2 and Wael M.K. Elfeil 4 (2020) Detection of Commercial Fraud in Processed Meat Products Using Rapid Techniques. *American Journal of Biochemistry and Biotechnology*, 16 (2): 244-251.

[9] Ghorban Elyasi Zarringhabaie1, Nasrollah Pirany2 and Arash Javanmard3 (2011) Molecular traceability of the species origin of meats using multiplex PCR *African Journal of Biotechnology* Vol.10 (73), pp. 15461-16465.

[10] Mehdizadeh M., Mousavi S. M., Rabiei M., Moradian K., Eskandari S., Abbasi Fesarani M., Rastegar H., Alebouyeh M. (2014). Detection of chicken meat adulteration in raw hamburgerusing polymerase chain reaction. *Journal of Food*

Quality andHazards Control. 1: 36-40.

[11] Al-taghlubee D. 1, Misaghi A. 1, Shayan P. 2,3 Akhondzadeh Basti A.1, Gandomi H. 1, Shayan D. 3,4 (2019) Comparison of Two Multiplex PCR Systems for Meat Species Authentication. *Journal of Food Quality and Hazards Control*. Vol. 6p. 8-15.

[12] Osman İrfan İLHAK1, Hüsnü Şahan GÜRAN2 (2015) Authentication of Meat Species in Sucuk by Multiplex PCR. *J. Fac. Vet. Med. Istanbul Univ.,*41(1), 6-11.

[13] Abdul-Hassan, I.A.1 and Tauma, J.A.2.(2014) Identification of Some Meat Species Using PCR and Multiplex PCR of Mitochondrial *Cytochrome B* Gene. *Iraqi Journal of Poultry Sciences.* Vol.8 No. (1) P. 1-9.

[14] Hassan, K.I. ; Ali B.A.M. and N.A. Mohammed (2019) Detection of the origin of animal species in kebab meat using mitochondrial DNA based - Polymerase Chain Reaction (mtDNA-PCR). *Iraqi Journal of Veterinary Sciences,* Vol. 33, No. 1, p. 39-43.

[15] Ding H. B. and Xu.R. J. (1999) Differentiation of Beef and Kangaroo Meat by Visible/Near-Infrared Reflectance Spectroscopy. *Journal of Food Science* Vol. 64, 814 – 817.

[16] Derya ARAÇ* Harun DIRAMAN Senem GÜNER. (2022) SOME CHROMATOGRAPHIC METHODS USED FOR SPECIES DETERMINATION IN MEAT AND MEAT PRODUCTS. *J. Halal & Ethical Res.* 4 (1): 62-70.

[17] Depamede. S. N. (2011) Development of a Rapid Immunodiagnostic Test for Pork Components in Raw Beef and Chicken Meats: a Preliminary Study. Media Peternakan, Agustus 2011, hlm. Vol. 34 No. 2. P. 83-87.

[18] Asensio, L., I. González, T. García and Martin, R. (2008) Determination of food authenticity by enzymelinked immune sorbent assay (ELISA). *J. Food Control*. 19:1–8.

[19] Eser İNCE, Nesrin ÖZFİLİZ (2018) Detection of adulterations in fermented and heat-treated Turkish type sausages by histological examination. *Ankara Üniv Vet Fak Derg*, 65, 99-107.

[20] Farag MR, Alagawany M, Abd El-Hack ME, Tiwari R, Dhama K (2015). Identification of different animal species in meat and meat products: trends and advances. *Adv. Anim. Vet. Sci*. 3(6): 334-346.

[21] Elena A. Zvereva 1 , Demid S. Popravko 1 , Olga D. Hendrickson 1, Natalia L. Vostrikova 2 , Irina M. Chernukha 2 , Boris B. Dzantiev 1 and Anatoly V. Zherdev 1,(2020) Lateral Flow Immunoassay to Detect the Addition of Beef, Pork, Lamb, and Horse Muscles in Raw Meat Mixtures and Finished Meat Products. *Foods*, 9, 1662.

[22] Esteki, M., Regueiro, M. and Simal-Gandara, J.(2019). Tackling fraudsters with global strategiesto expose fraud in the food chain. Comprehensive Reviews in *Food Science and Food Safety*18: 425-440.

[23] Arslan, A., I. Ilhak, M. Calicioglu and M. Karahan. (2005) Identification of meats using random amplified polymorphic DNA (RAPD) technique. *J. Muscle Foods*. 16(1): 37–45.

[24] SAFIYYAH SHAHIMI, SAHILAH ABD. MUTALIB*, WAN SAKEENAH WAN NAZRI, AMINAH ABDULLAH & NORRAKIAH ABDULLAH SANI (2018) Comparison of DNA Profiling between Fishes and Pork Meat using Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLP) Analysis. *Sains Malaysiana* 47(7) :1535–1540.

[25] Yong Hyun Park1,2, Md. Rasel Uzzaman3, Jeong-Woon Park2, Sang-Wook Kim3,Jun Heon Lee1, and Kwan-Suk Kim3.(2013) Detection of Meat Origin (Species) Using Polymerase Chain Reaction. *Korean J. Food Sci. An.,* Vol. 33, No.6,pp.696-700.

[26] Chen Chen,1,2 Jia Chen,1 Yan Zhang,2 Yongbo Li,2 Zan Wang,2 Xian Li,2 Wei Zhou,2,3 and Zhisheng Zhang.(2020) Quantitative Detection of Beef and Beef Meat Products Adulteration by the Addition of Duck Meat Using Micro Drop Digital Polymerase Chain Reaction. *Journal of Food Quality* Volume 2020, Article ID 2843056, 8 pages.

[27] Aina GQ, Erwanto Y, Hossain M, Johan MR, Ali MdE, Rohman A (2019) The employment of q-PCR using specific primer identification of wild boar meat in meatball samples. *Journal of Advanced Veterinary and Animal Research*. Vol. 6: 300-7.

[28] Muhammad Sulyman Saleem (2022) TRIPLEX PCR ASSAY FOR IDENTIFICATION OF HALALMEATSPECIES. *J. Agric. Res.,* Vol. 60(2):153-159.

[29] Sangthong, D. 1, Suwannarat, S. 2, Samipak, S. 2 and *Sangthong, P. 2 (2021) Multiplex PCR assay for species identification of meat and dairy products from buffalo (*Bubalus bubalis*), cattle (*Bos indicus* and *Bos taurus*), goat (*Capra hircus*), and sheep (*Ovis aries*). *International Food Research Journal* 28(4): 716 - 725.

[30] Andrejevic M, Markovic, MK, Bursac B, Mihajlovic M, Keckarevic D (2019) Identification of a broad spectrum of mammalian and avian species using the short fragment of the mitochondrially encoded Cytochrome b gene. *Forensic*

Science, Medicine and Pathology. 15:169–177.

[31] Prusak B, Grzybowski G, Zieba G (2004) Taxonomic position of *Bison bison* (Linnaeus, 1758) and *Bison bonasus* (Linnaeus, 1758) based on analysis of *cytb* gene. *Animal Science Paper and Reports*. 22: 27–35.

[32] Kim MJ, Suh S. M., Kim SY, Qin P, Kim HR, Kim HY (2020). Development of a Real-Time PCR Assay for the Detection ofDonkey (*Equus asinus*) Meat in Meat Mixtures Treated underDifferent Processing Conditions. *Foods* 9: pii: E130.

[33] Mayada R. Farag1*, Khlood M. El Bohi1, Samah R. Khalil1, Mahmoud Alagawany2*,Muhammad A. Arain3, Khan Sharun4, Ruchi Tiwari5, Kuldeep Dhama6. (2020) FORENSIC APPLICATIONS OF MITOCHONDRIAL CYTOCHROME *B* GENEIN THE IDENTIFICATION OF DOMESTIC AND WILD ANIMAL SPECIES. *Journal of Experimental Biology and Agricultural Sciences*, February - 2020; Volume 8 (1) page 1 – 8.

[34] Pal, M., Ayele, Y., Patel, A. S., and Dulo, F. (2018). Microbiological and hygienic quality of Meat and Meat Products. *Beverage Food World*, 45, 21-27.

[35] Kahl, B. C., Becker, K., &Löffler, B. (2016). Clinical significance and pathogenesis of staphylococcal small colony variants in persistent infections. *Clinical microbiology reviews*, 29(2), 401-427.

[36] Abdel-Atty, N. S., Khalafalla, F. A., &Barakat, D. A. (2020). Bacteriological Quality of Canned Meat Marketed in Egypt. *Benha Veterinary Medical Journal*, 39(1), 154-158.

[37] Shayan P., Al-Taghlubee D., Misaghi A., Shayan D., Gandomi H., heAarunOukABasti A., Alghassab T., Kamkar A., Khanjari A., Eckert B. (2018). An innovative reverse line blot for simultaneous detection of animal species in food. *European Food Research and Technology*. 244: 1711-1717.

[38] Hamouda Ahlam F.1, Eltanani Gehan SA.2 and Radwan Mervat I.3(2020) Detection of Meat Products Adulteration by Polymerase Chain Reaction (PCR) Assay in Kalubia Governorate, *Egypt. Ann Clin Med Res.* Vol. 1(3): 1015.

[39] Siriken, B. (2004). The microbiological quality of ground beef in Aydin and Afyon Provinces, Turkey. *Revue Méd.Vét*, 155(12), 632-6.

[40] Mafra I, Ferreira IMPLVO, Oliveira MBPP. (2008) Food authentication by PCR based methods. *Eur Food Res Technol*. 227(3):649-65.

[41] Abbas O, Zadravec M, Baeten V. (2018) Analytical methods used for the authentication of food of animal origin. *Food Chem*. 246:6-17.

[42] Tibola, C. S., da Silva, S. A., Dossa, A. A. and Patricio, D. I. (2018). Economically motivated food fraud and adulteration in Brazil: incidents and alternatives to minimize occurrence. *Journal of Food Science* 83: 2028-2038.

[43] Poonia, A., Jha, A., Sharma, R., Singh, H.B., Rai, A.K. and Sharma, N. (2016). Detection of adulteration in milk: a review. *International Journal of Dairy Technology* 69: 1-20.

[44] Tanabe E, Miyauchi A, Muneshige K, Mio C Sato, Sato M (2007). PCR method of detecting pork in foods for verifying allergen labeling and for identifying hidden pork ingredients in processed foods. *Biosci. Biotechnol. Biochem.*, 71: 1663-1667.

[45] Yosef, T., M. Al-Julaifi and A. Al-Rizqi, (2014). Food forensics: Using DNA-based technology for the detection of animal species in meat products. *Nature Sci.*, 12: 82-90.

[46] Muangkram Y., Wajjwalku W., Amano A., Sukmak M. (2018) The novel primers for mammal species identification-based mitochondrial cytochrome b sequence: implication for reserved wild animals in Thailand and endangered mammal species in Southeast Asia. Mitochondrial DNA Part A, DNA mapping, sequencing, and analysis 29:62–72.

[47] Yacoub HA, Fathi MM, Mahmoud WM (2013) DNA barcode through *Cytochrome b* gene information of mtDNA in native chicken strains. Mitochondrial DNA 24 : 528-537.

[48] Hadj-Henni L, De Meulemeester T, Depaquit J, Noel P, Germain A, Helder R, Augot D (2015) Comparison of Vertebrate Cytochrome b and Prepronociceptin for Blood Meal Analyses in Culicoides. *Frontiers in Veterinary Science* 2: 15.

[49] Tobe, S. S. and Linacre A. M. T. (2008). A multiplex assay to identify 18 European mammal species from mixtures using the mitochondrial *cytochrome b* gene. *Electrophoresis* 29:340–347.

[50] Lanping WANG1, Xinru HANG2, Rongqing GENG2*(2019) Molecular detection of adulteration in commercial buffalo meat products by multiplex PCR assay. *Food Sci. Technol,Campinas,*Vol.39No.(2):Pages344-348.

[51] - Javed A (2016). Food Borne Health Issues and Their Relevance to Pakistani Society. *American Scient. Res. J. Engin. Technol. Sci*. (ASRJETS). 26(4): 235-251.

[52] AttAullAh Bughti1, ShAhid huSSiAn ABro1*, ASghAr Ali KAmBoh1, riAz Ahmed leghAri2, ChAndAr KumAr3,

ShAfique Ahmed KoondhAr1. (2017) Bacterial Contamination of Raw Meat and Butchers' Equipment in Retail Shops in Tando-Allahyar, Pakistan. *Journal of Animal Health and Production*. Volume 5 | Issue 3 | Page 116.

[53] Ebovitz, E., Evans, J. B., & Niven J. R., C. F. (1955). Tellurite-glycine agar: a selective plating medium for the quantitative detection of coagulase-positive Staphylococci. *Journal of Bacteriology*, 70 (6), 686-690.