IDENTIFICATION AND DIFFERENTIATION OF POULTRY MEAT AND PRODUCTS USING PCR-RFLP TECHNIQUE

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ABSTRACT

The mitochondrial cyt b gene plays a serious role in investigating untruthful meat species. This study aimed to authenticate the species of poultry products (Escallop, Nugget, Steak, and Sausage) depending on cyt b gene by using universal cyt b primer. DNA was isolated, and then a band of 359 bp of a mitochondrial cytochrome b gene was produced during the PCR amplification. The PCR products were exposed to Hinf1 and Rsa I restriction enzymes. The restricted fragments produced by restriction fragment length polymorphism technique (RFLP), were run by agarose gel electrophoresis. Results showed that all products had a similar band except sausage product does not follow the rule and showed mislabeling product by the REs, Two bands were yielded by HinfI RE for all products (114 and 245) bp with the differentiated sausage among other products based on the fake product (63 and 296) bp, while digestion by Rsa I produced three bands for escallop, nugget, and steak, (63, 100, 196), but only two bands for sausage was generated (148 and 211). As result, the study offered that analyzing meat products to detect the origin species via a PCR-RFLP technique by using these restriction enzymes can give reliable results. In short sausage is considered as fraud products because the results showed different bands as compared with poultry meat.

Keywords: RFLP-PCR; Poultry meat Identification; Mt cyt b gene; Restriction Enzyme.

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INTRODUCTION

In most regions of the world, meat and their products intake carry on to increase and particularly in developing nations (cawthorn et al., 2013 and keyvan et al., 2017). Meat has always been eaten by people in different types, both after thermal processing or prepared in a way that requires long-term packing, such as dry sausages ….etc. These based products have become rare and expensive throughout the year, they are sold on the market at parentally excessive expenses, and for this purpose they are desirable goals for the meat commercial fraud. Furthermore, it is a critical part of the administrative problem of sustenance as tainted and substitution of meat has been a reliable concern for various reasons, for example, community health, religious aspects, future health and unwanted task in meat markets (rashid et al., 2014; hou et al., 2015 and farag et al., 2015).

In order to protect clients and avoid unfair opposition, the detection and prevention of deceptive practices requires easy and rapid research methods that are appropriate for routine applications of these products. Different approaches based on the study of
species-specific components consisting of protein and DNA have been established to
distinguish meat and their products originating from different species of animal and to
prevent fraudulent practices. It is possible to classify organisms by analyzing proteins
via various methods using immunological, chromatographic and electrophoretic
methods (Kang’ethe et al., 1986; Berger et al., 1988; Zerifi et al., 1992; Amstrong and
Leach, 1992 and Gallardo et al., 1995). Nevertheless, even in the identical species,
protein denaturation in meat during warmth treatment or other technological process
and variation in protein composition decreases these methods. In addition, these
techniques may be insufficient to discriminate between species that are closely related
and not suitable for routine use, as it is difficult and time consuming to isolate species-
specific protein. Moreover, these techniques can be insufficient to differentiate between
species that are closely related and are not appropriate for using routinely, as the
isolation of species-precise proteins is hard and time-consuming as it is tough and labor
to isolate of species-specific proteins is (Hofmann, 1987; Jemmi and Schlosser, 1992
and Koh et al., 1998 and Kesmen et al., 2010).

Various molecular approaches have been developed to recognize origin meat
species. These methods can reduce the insufficiencies of common techniques (Girish et
al., 2005). PCR, AFLP, RAPD, DNA hybridization and RFLP are included in molecular
markers (Rodriguez et al., 1991; Arslan et al., 2005 and Alves et al., 2002). Polymerase
chain response-based methods are outstandingly fast and reliable, and presently they
have proved to be a standard for meat distinguishable confirmation in the industry
(Kesmen et al., 2010). Particularly mtDNA has been the foremost studied eukaryotic
genomes district which has expected an essential work being created of population and
developmental hereditary qualities (Abou-Hadeed et al., 2011 and De Masi et al., 2015).
The gene cytochrome b (cyt b) expresses one of the best-known proteins that make up
the mitochondrial phosphorylation matrix complex III and is the only one expressed by
the mitochondrial genome. The cyt b gene is used in valid drug and molecular evolution
research as a critical utility tool (Prusak et al., 2004; Al-Sanjary, 2009; Abou-Hadeed et
al., 2011 and Farag et al., 2015).

The aim of the study was to authenticate and detect commercial fraude in certain
meat products such as escallop, nugget, steak and sausage obtained from different
markets in Erbil-Iraq and the results will be used to compare labels written on products
resulting from chicken meat, whether true or not, using the PCR-RFLP molecular
technique for mitochondrial cytochrome b gene analysis, using 2 restriction enzymes
(Hinfl and Rsa I). These restriction enzymes have not been used for identification before
in Kurdistan region- Iraq.

**MATERIALS AND METHODS**

**Sample Preparation and DNA Isolation**

This study was done on meat of four types of poultry products (Escallop, Nugget, Steak
and Sausage), in laboratory of molecular genetics in Salahaddin University- Erbil,
college of agricultural engineering sciences and a laboratory in genome company in
Erbil. Ten samples in each type were collected from different markets in Erbil
governorate (mixed together to make 4 main samples as a polled sample from same
origin), and chicken meat in different parts (breast, wing, leg) was taken as used as a positive control for comparisons.

Meat samples of the concerned products were stored in aluminum foil at -20 °C (until all the samples were collected for about 2 weeks) for DNA extraction. DNA was extracted by using Blood-Animal-Plant DNA Preparation Kit (Spin column based genomic DNA purification, Jena Bioscience GmbH, Germany) according to manufacturer's instructions. The isolated DNA was labeled and stored at -20 °C for the next stage. The purity of DNA was checked by Nanodrop spectrophotometer (Thermo scientific UK) and gel electrophoresis.

**PCR Primers**

Polymerase chain Reaction (PCR) occurred utilizing a modification of the forced restriction fragment length polymorphism (RFLP) strategy. The primer sequences used for this research were showed in Table 1.

**PCR Amplification**

The target DNA (mtcyt b gene, forward and reverse primers) for each species was amplified by PCR (Applied Biosystems® Veriti® 96-Well Fast Thermal Cycler, USA). The final reaction volume for each one was of 25µl. The PCR component for amplification of cyt b gene is shown in Table 2. The cycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles consisting of denaturation at 95°C for 0.30 min, annealing 50°C 1 min, and extension at 72°C for 45 sec, with final extension 72°C for 7 min then holded at 4 °C for infinity. The PCR products were screened in a 2% agarose gel containing ethidium bromide (LOT:110802BB197, Bio Basic Inc.) in Tris-borate EDTA buffer and visualized under UV transillumination (Biostep-UST-20M-8K).

| Table (1): Sequence of cyt b primers |
|-------------------------------------|
| **Gene name** | Nucleotide Sequences | amplified size | Reference |
|---------------|----------------------|----------------|-----------|
| Cyt b (NP_904340.1, gene ID: 17711) | F: 5'CCATCCAAACATCTCAGCATGATGA AA-3' R: 5'-GCCCCTCAGAATGATATTGTTGCTCTCA-3' | 359 bp | Meyer *et al* (1995) |

**RFLP Analysis**

The obtained PCR products were digested by exposing to the selected Hinfl 5’-G/ANTC-3’ / 3’ – CTNA/G-5’ and Rsa I 5’ GT/AC-3’ / 3’- CA/TG-5’ (ADR6201 00001211493, Promega- USA) restriction enzymes for each PCR products separately.
The digestion mixture consists of (2 µl of 10X Reaction buffer, 0.5 µl (5 U) of Reaction Enzyme, 10 µl PCR product and filled with 7.5 µl of free deionized water to complete the final volume 20 µl). This mixtures were incubated from 2 to 4 h, (37 °C) according to the restriction enzymes manufacture instructions. 10µl of the digested samples were loaded, in 2.5% agarose gel. The length of the fragments produced in digestion was matched with the 100 bp DNA Ladder RTU (Cat NO. DM012-R500, Promega- USA).

RESULTS AND DISCUSSION

Mitochondrial DNA was isolated successfully and Purity of DNA ratio ranged from 1.7 to 1.9. The universal cytochrome b gene was clearly produced 359bp as shown in figure 1, with no differences for each sample when run in 2% agarose gel. Then amplicons were exposed to restriction enzymes Hinfl and Rsa I, different positions of amplified DNA were cut by these enzymes. Formerly, digested fragments were separated by 3% agarose gel. The samples displayed different fragment sizes, as shown in Table 3 and Figure 2, 3, when they were imaged via UV Transilluminator and linked with the standard size ladder for comparisons.

Table (3): Number of bands and their sizes of poultry products produced by (Hinfl, RsaI) REs.

| Poultry Products | *Hinfl* fragment size pb | *RsaI* fragment size pb |
|------------------|--------------------------|-------------------------|
| Escallop         | 2 bands (245,114)        | 3 bands (63, 100, 196)  |
| Nugget           | 2 bands (245,114)        | 3 bands (63, 100, 196)  |
| Steak            | 2 bands (245,114)        | 3 bands (63, 100, 196)  |
| Sausage          | 2 bands (63,296)         | 2 bands (148,211)       |

Figure (1): PCR product of poultry products, Escallop, Nugget, Steak and Sausage and chicken meat (control).
Figure (2): Digestion of PCR product of poultry products, Escallop, Nugget, Steak and Sausage and chicken meat (control) with HinfI restriction enzyme of Cyt b gene fragments.

Figure (3): Digestion of PCR product of poultry products, Escallop, Nugget, Steak and Sausage and chicken meat (control) with RsaI restriction enzyme of Cyt b gene fragments.

In comparison, identification the source of meat and the products by molecular techniques offer satisfied and optimistic result over traditional morphological or protein identification and so on, for that reason DNA based methods are the best method for identifying species in both commercial foods and animal products (Ali et al., 2015; Andrea et al., 2015). Currently, meat adulteration has become a common in many poor countries, therefore it is vital to identify and confirm the commercial fraud meat and products for the public, which cannot be observed with touching or sensing organism, and discover the origin species for those products. Because it is critical for food safety, consumer demands, and law. For these using PCR based method is very trustworthy for the quality of food generally and the meat products specifically (Al-Sanjary, 2009; Farag et al., 2015, Khan et al., 2018 and Dilger et al., 2020). As a result of both restriction enzymes that were done all samples separately at the same time, we can notice that the sausage was displayed mislabeling and marked as fraudulent because was not parallel to the chicken meat (positive control) and chicken meat was not used during the process of sausages. Although it was labeled as a chicken meat and there was possibly other meat species that may be replaced with the original meat.

Studying the mitochondria cytochrome b gene via RFLP-PCR based method shown brilliant power and actual tool for separating all products samples such as (Escallop, Nugget, Steak and Sausage) because there were no unclear amplified fragments when cleaved with restriction endonucleases among them. Hence, restriction enzymes offered
exact bands for all samples and there was no need to use statistical analysis to identify significant or nonsignificant among the samples. It is more probable that meat of two or more species is mixed for fraudulent purpose during meat processing such as grinders, cutters, knives, choppers. In order to detect traces of meat mixtures samples have to be kept for PCR-RFLP method due to is a very powerful method (Khan et al., 2018). The results were achieved in utilizing the HinfI, RsaI restriction enzymes absolutely recommended that these results were suitable to authenticity and demonstrate unreal meat source and proposed to utilize other types to detect the real sausages species.

In general, meat identifications previous Study was done based on tissues and mixed processing (Ong et al., 2007, Ghovvati et al., 2009 and Ciupa et al., 2012), while in this paper focused on products in general which were commonly used for human daily nutrients, that labeled as a chicken meat, so that the obtained results were a vital and established a very effective determination in all used products by cyt b mt DNA. Moreover, this research approved that there was a satisfactory level of the aimed DNA to amplify in a PCR. Then this is a confirmatory point to settle the benefits of utilizing the mitochondrial DNA more than nuDNA (Pakendorf and Stoneking, 2005). Ayaz et al. (2006) stated that 11 of 28 (39.2%) sausage testers that were affirmed as beef were established mixing beef and poultry meat together. Also, the results were agreement with the research done by (Keyvan and coworker, 2017) that detected the adulteration with sausage products. Kesmen and colleagues (2007) used species-specific primers for identification of horse, donkey, pig, beef and sheep DNA in sausage for amplification of various parts of mt DNA, and then they observed one in a hundred ng DNA in every sample.

**CONCLUSION**

The current study revealed that the RFLP-PCR undeniably is a reliable and prevailing method for detection species meat from Fraud Products beside that using the mitochondrial cytochrome b gene, with the help of two restriction endonucleases (HinfI and RsaI) can be very effective and powerful. Moreover, it is a rapid technique and does not need hardworking and inexpensive method. This research indicated that, the meat source that used to make sausage is not produced with chicken meat, and does not obey the public health, low as well, which offered to a public as a chicken meat product. A fraudulent product in any country is a risk and protection are a must. To keep public health and avoid fake products meat products must be frequently analyzed by quality control in governmental organizations.

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**DISCLOSURE STATEMENT**

The authors declare that they have no conflicts of interest.
تحديد وتمييز لحوم الدواجن ومنتجاتها باستخدام تقنية تباين أطوال قطع التقييد - تفاعل البلمرة المتسلسل

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الخلاصة
يلعب جين cyt b في المايتوكوندريا دورا مهما في التحديد انواع لحوم الغير الصحية. هدفت الدراسة الحالية الى التحقيق من انواع منتوجات لحوم الدواجن (Escallop, Nugget, Steak, Sausage) باستخدام البادئ المعروف عالميا cyt b. تم عزل الحمض النووي DNA ومن ثم تضخيم قطعة DNA قاعدة زوجية في تقنية التفاعل بالبلمرة المتسلسل PCR. ثم تم هضم القطع المضخمة مع الأنزيمات القاطعة Hinf1 و Rsal بواسطة الترحيل الكهربائي بالهلام (agarose). أظهرت النتائج أن جميع المنتجات لها نفس الشروط الانتقائي استثناء منتج Sausage الذي لم يتبوع الشروط وكانت تسميتها المنتوج خاطئة بالاعتماد على الأنزيمات القاطعة، حيث تم انتاج قطعتين 114 و 296bp بينما كانت القطعتين 63 و 245bp لكل من Escallop و Nugget و Steak. في حين انتجت النتيجة كاشفة في المنتج Sausage المختلفة لمنتج Sausage بوسطة إنزيم القطع Hinf1، حزم (63, 100 و 196bp) فاقيا زوجية لجميع المنتجات عدا منتج Sausage الذي أظهر ب /><148 و 211bp فاقيا زوجية. استنادا على النتائج هذه الدراسة، فإن استخدام تقنية PCR-RFLP يمكن أن يعطي نتائج موثوقة لتحليل وكشف عن أصل منتجات اللحوم. بالقصير، Sausage يعتبر من المنتجات المغشوشة، لأن النتيجة تظهر الحزمة المختلفة إذا قارن مع الحزمة اللحوم الدجاج.

الكلمات المفتاحية: PCR-RFLP، تحديد لحوم الدواجن، الجين المايتوكوندريا سايتوكروم بي، الأنزيمات القاطعة

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