Detection of the origin of animal species in kebab meat using mitochondrial DNA based - Polymerase Chain Reaction (mtDNA-PCR)

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(Received April 22, 2018; Accepted August 17, 2018)

Abstract

Adulteration and incorrect labeling of meat products became a matter of great concerns of religious, economical, legal and medical aspects. Among meat products, kebab is considered one of the most favorable in Iraq, which makes it prone to adulteration. This study was carried out to evaluate the quality of kebab by detecting the origin of animal species meat in it; using mitochondrial DNA (mtDNA) based Polymerase Chain Reaction (PCR) under laboratory conditions. For this purpose, kebab was prepared as per the standardized processing schedule using pure mutton and beef meat in ratio 80:20 and chicken meat in five ratios 70:20:10, 65:20:15, 60:20:20, 55:20:25 and 50:20:30, respectively. DNA was extracted successfully from pure species meat and from all mixed kebab above, then PCR was carried out using species-specific primers, to amplify mitochondrial cytochrome b (cyt b) gene. The results revealed specific amplified fragments with 133, 300 and 585 bp for pure chicken, beef and sheep, respectively, and in the mixed grilled samples, the detection limit of chicken was 10%, indicating that the cooking (grinding) and addition of non-meat ingredients showed no effect on the detection of meat species. The results of this study proved mtDNA-PCR to be effective and reliable for detecting the origin of animal species meat. This method of detection could be applied in quality control laboratories for detect adulteration in different kinds of traditional grilled kebab in Iraqi restaurants.

***Keywords**:* Kebab, animal origin, Detection, mtDNA, PCR
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الكشف عن نوع لحم الحيوان المستخدم في الكباب باستخدام تفاعلات تضاعف الدنا المتسلسل المعتمد على دنا الميتوكوندريا (mtDNA-PCR)

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الخلاصة

نال الغش والتعليم غير الصحيح لمنتجات اللحوم اهتماما كبيرا من النواحي الدينية والاقتصادية والقانونية والطبية. ومن ضمن منتجات اللحوم، يعد الكباب من أكثر تلك المنتجات تفضيلاً في العراق، مما يجعله عرضة للغش. تم إجراء هذه الدراسة لتقييم جودة الكباب من خلال الكشف عن نوع لحم الحيوان فيه وذلك باستخدام تفاعلات تضاعف الدنا المتسلسل المعتمد على دنا الميتوكوندريا تحت ظروف المختبر. ولتحقيق هذا الهدف تم إعداد الكباب اعتمادا على النسب القياسية المعتمدة في الأسوق العراقية وذلك باستخدام لحم الضأن واللحم البقري بنسبة ٨٠:٢٠، إذ تم إضافة لحم الدجاج في خمس نسب بتقليل نسبة لحم الضأن ورفع نسبة لحم الدجاج وكالاتي ٧٠:٢٠:١٠، ٦٥:٢٠:١٥، ٦٠:٢٠:٢٠، ٥٥:٢٠:٢٥، ٥٠:٢٠:٣٠ على التوالي. تم استخراج الدنا بنجاح من انواع اللحوم بشكلها النقي وكذلك من خلطات الكباب وبالنسب أعلاه، بعدها تم تنفيذ تفاعلات تضاعف الدنا المتسلسل باستخدام بادئات خاصة بالأنواع، لتضخيم جينات السيتوكروم ب، كشفت النتائج عن الحصول على قطع متضاعفة بحجم ١٣٣، ٣٠٠، ٥٨٥ متخصصة للحم الدجاج ولحم البقر والأغنام، على التوالي، بشكل نقي اولا وفي العينات المختلطة للكباب، تم الكشف عن نسب الدجاج حتى بتركيز ١٠ ٪ والذي يدل على عمليه الطهي

(الشوي) للكباب لم يكن لها أي تأثير على عملية الكشف عن أنواع اللحوم. وبهذا أثبتت نتائج هذه الدراسة أن تفاعلات تضاعف الدنا المتسلسل المعتمد على دنا الميتوكوندريا كانت فعالة وموثوقة في الكشف عن نوع لحم الحيوان المستخدم في اعداد الكباب والذي يمكن تطبيقه في مختبرات مراقبة الجودة للكشف عن الغش الذي يحصل في هذا المنتوج في المطاعم العراقية عند استخدام انوع رخيصة أو محرمه من اللحوم.

Introduction

The food industry provides the market with an increasing number and variety of raw, cooked and ready-to-eat food products with different preparation methods using different food additives such as spices, salts and sugar. Meat products are considered to be a rich source of nutrients including protein, minerals, vitamins, poly unsaturated fatty acids, iron, zinc, and many micronutrients (1). The high demand on meat and meat products and their height cost makes them prone to adulteration. The act of intentionally substituting of one costly meat species with another cheaper one or using other meat species with false labels make it an important task for food control to be able to carry out species differentiation of the raw materials to be used for industrial food preparation and to detect animal species in food products (2). Meat species specification is an area which needs specialized attention in the food quality management system, it is a vital field to ensure the food safety to the consumers and it conserves the laws related to meat and meat products. The ability to detect objectionable species in meat products is important not only for economic, health, religious and ethical reasons, but also to ensure fair trade and compliance with legislation (3). Various methods have been applied for meat species specification; the physico-chemical methods gave idea about meat species based on the amount of certain chemicals present in meat of different animal species, but the reproducibility of these methods was not possible, and cannot be applied on processed meat and meat products (4), Furthermore the immunological techniques such as enzyme-linked immune sorbent assays (ELISA) were also used for meat species specification (5), but the available antisera shows cross reactions and were not effective on processing food, hence the immunological techniques face difficulty in distinguishing closely related species which hampers their effectiveness. Methods that based on protein analysis have also been applied for meat fraud identifications (6). The disadvantages of these methods are being time consuming, expensive and are not specific enough. Recently, DNA-based techniques, used for such purposes revealing high specificity, accuracy, reliability and legal acceptability, besides they are fast and inexpensive (7,8). Moreover DNA are relatively stable at high temperatures, meaning that it can be analyzed not only in fresh and frozen food products, but also in processed, degraded and mixed commodities (9), hence, DNA based techniques are considered as the most appropriate methods for species detection and identification in processed foods (4). Among DNA based techniques Polymerase Chain Reaction (PCR) methods deserves special attention due to its high sensitivity and specificity as well as relatively short period of time necessary to perform the analysis. There are many types of PCR, which almost differ in the kind of primers they used. Specie Specific PCR means application of PCR on a species using specific primers that target sequence of DNA which are unique and specific to that species (10). It was used for identification of many animal species in meat products even in cooked meat under different processing conditions (11). The sequence of the DNA that have been targeting by PCR, may be nuclear DNA or mitochondrial DNA, both have been used, for identification of meat species. The target nuclear DNA's include: porcine leptin Gene (12), bovine beta-actin gene (13), whereas the target mitochondrial DNA's include: 12S rRNA (14), D-loop region (15) and cytochrome-b gene (16). The cytochrome b gene of mtDNA was used to distinguish different types of meat like chicken, turkey, beef, pork, lamb, duck, goose, pheasant, quail and guinea fowl in meat products (17-20). In Iraq one of the most favorite meat products is kebab (21) which consists of mixed ground beef, mutton or goat meat with some of spice, salt, flour and chopped onion. Kebab is prone to adulteration using cheaper kinds of meat, hence the present study was carried out to evaluate the quality of kebab meat by detection of chicken meat using mitochondrial DNA based Polymerase chain reaction ((mtDNA-PCR) method.

Materials and methods

Sampling

Fresh lean beef (*Bos taurus*), sheep (*Capra hircus*) and chicken (*Gallus gallus*) were purchased from a local market in Sulaymaniyah - Iraq. The meat of each sample was minced separately to avoid cross contamination. The kebab was prepared according, to standard processing schedule using pure mutton and beef meat in ratio (80:20), then chicken meat was added in five ratios 70:20:10, 65:20:15, 60:20:20, 55:20:25 and 50:20:30 respectively. A total of 50g of meat samples from individual species and 100 g of mixed meat samples were collected to make kebab. The samples so obtained were packaged in properly labeled LDPE bags. These mixtures were used as a reference material for DNA extraction.

DNA extraction

The DNA was isolated from pure species and from the mixed meat using two methods (i) Kit (Gene aid, Korea) and (ii) phenol-choloroform-isoamil method (22) as follows: meat sample (300 g) was cut and homogenized using a knife and milled with mortar and pestle and liquid nitrogen. The homogenized sample was transferred into a sterile 15 ml tube and 500 µl of lysis buffer (NaCl, 50 mM; EDTA pH 8, 25 mM; Tris-HCl pH 7.6, 50 mM) and 20 µl of proteinase K (10 mg/ml solution). The solution was incubated in a rotated stirrer at 56 °C for 60 min, then 500 µl of phenol: chloroform: isoamylalcohol (25:24:1) was added to each microtube and mixed gently for 5 min. The final suspension was centrifuged at 10000 x g for 5 min. The aqua phase of each sample was carefully transferred to a new sterile 1.5 ml microtube, and then 500 µl chloroform was added to each sample. Centrifugation was repeated similar to the above conditions for 1 min. The aqua's phase was transferred to a new sterile 1.5 ml microtube. and 1 ml of cold isoproponal with 50 µl of 3 M sodium acetate was added and the suspension was incubated on ice for 30 min. Subsequently, the suspension was centrifuged at 10000 xg for 10 min at 4 °C. The supernatant containing alcohol was removed and the DNA pellet was washed with 250 µl cold ethanol (70%). The concentration and quality of the obtained DNA was determined by measuring the absorbance at 260 nm. DNA quality (purity) was determined by measuring the absorbance at 260- 280 nm. The extracted DNA was stored at -20 °C for further analysis.

Amplification of cytochrome b gene

PCR amplification was performed using Master mix (Genet Bio, South Korea) containing a total volume of 12.5 µl reaction mixture (1U of *Taq* DNApolymerase, 10 mM of Tris-HCl (pH9.0), 30 mM of KCl, 1.5 mM MgCl2, 0.5 mM each dNTPs (dATP, dCTP, dGTP, dTTP), 2 µl of DNA template (25-50ng) and 2 µl primer (Genet Bio, Korea). Table 1 illustrated the information about the sequence of the primers used in this study. For DNA amplification, the following PCR program was used: One step of 2 min at 94 ºC; 30 cycles, with one cycle consisting of 30s at 94 ºC, 60 s at 60 ºC (for sheep and beef specific primers) 58 ºC (for chicken primers), and 60 s at 72 ºC; and one step of 5 min at 72 ºC. The PCRs were performed with the thermo cycler (MWG Biotech, UK). In order to evaluate and verify the specificity of PCR protocol for each species, the primer pairs were tested by PCR on DNA templates prepared form other kinds of species. The products of PCR reaction were run on 1.2% agarose gel electrophoresis and stained by ethidium bromide to visualize the amplified fragments under UV.

Table 1: The sequence of primers for each type of animal studied with the molecular size of the expected amplified product

Species	Primer Sequences (5'-3')	Expected size (bp)	Reference
Sheep	Forward: TAC CAA CCT CCT TTC AGC AAT T Reverse: TGT CCT CCA ATT CAT GTG AGT GT	585	Zarringhabaie *et al.* (23)
Chicken	Forward: AGC AAT TCC CTA CAT TGG ACA CA Reverse: GAT GAT AGT AAT ACC TGC GAT TGC	133	Bo *et al.* (24)
Beef	Forward: CAA TAA CTC AAC ACA GAA TTT GC Reverse: CGT GAT CTA ATG GTA AGG AAT A	300	Kotowicz *et al.* (25)

Results and discussion

In order to evaluate and verify the specificity of the species-specific primers in this study, each pair of primers was performed at first in PCR reaction using the DNA extracted from the meat of pure individual species (chicken, beef and sheep). The reaction generated specific fragments, as the electrophoresis indicated single specific band with the expected size of 133, 300 and 585 bp for chicken, beef and sheep respectively, and no fragment, was produced by non-specific amplification. To evaluate the specificity of the chicken primers, the PCR was carried out on the DNA extracted from the kebab mixed samples (Figure 1) and results expected an amplified product (133 bp) for all mixtures that contain chicken, with different concentration even in the ratio of 10 %. However, more intense bands were seen as the rate of chicken meat was increased (Lane 5 and 6 in figure 1). The chicken specific primer was used for the detection of chicken meat among other meat products by Kitpipit *et al*. (26), Bo *et al*. (24). The successful detection of this target fragment using PCR may be due to the small size of amplicon (133bp). Previous reports have also shown that amplicon size of less than 150 bp has a higher chance of survival in cooked samples (15), as the cooking causes extensive changes in the meat tissue during heat treatment which make the speciation becomes difficult in cooked samples.

PCR amplification using beef specific primer (Figure 2) produce amplified product with 300bpp and with approximately the same intense bands for all of mixtures, due to the equal ratio of beef meat (%20) in all of the mixtures. Beef specific primer was also used by Mehdizadeh *et al*. (27) and Kotowicz *et al.* (25) for the detection of beef meat using PCR techniques. In application

of PCR using the sheep specific primers (Figure 3), amplified product with the expected size (585 bp) produced in all the mixtures with a decrease in the intensity of the bands due to the decrease in the concentration of sheep meat from 70% to 30%, sheep specific primers were also used by Zarringhabaie *et al*. (23) and also by Bhat *et al*. (28) for detection of sheep meat in processed food using PCR.

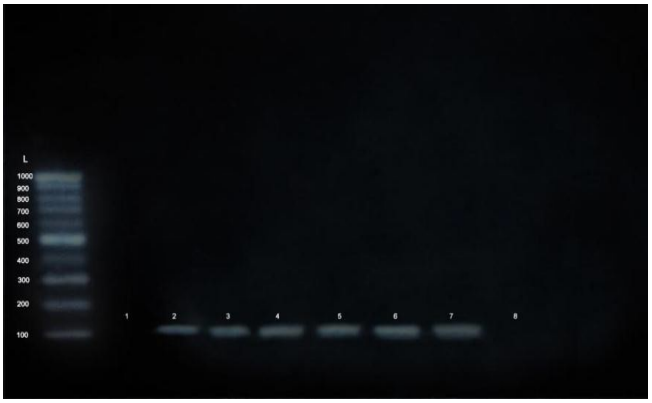

Figure.1: PCR product with 133 bp using chicken specific primer in 1.2% agarose gel electrophoresis. lane 1 represent DNA ladder, lane1 have no chicken meat, lane 2;10% chicken meat, lane 3: 15% chicken meat, lane 4; 20% chicken meat, lane 5; 25% chicken meat, lane 6; 30% chicken meat, lane 7; positive control and lane 8; Negative control.

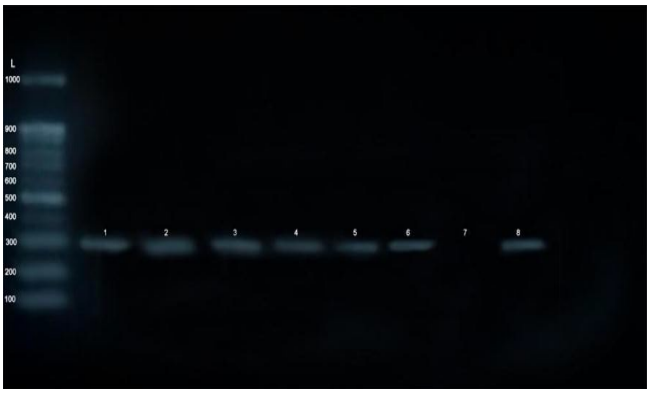

Figure.2: PCR product with 300 bp using Beef specific primer in 1.2% agarose gel electrophoresis, lane L; DNA ladder, lane (1-6) contain 20% of beef meat, lane 7; negative control and lane 8; positive control.

Consequently, the Mitochondrial DNA (mtDNA) based PCR procedure used in this study proved to be effective and reliable in detecting the animal origin of kebab meat. The reason for using mitochondrial DNA for the detection of animal species in any meat product is due to its high copy number which give high chances for their survival and less chances of degradation under different meat processing conditions thereby making it ideal for meat species identification in processed samples (29,30). This assay has many other applications in identification of fraud meat and meat products, such as the detection of foreign or undesired or forbidden animal species in commercial meat products. It is suggested that this method of detection can be applied to quality control to detect the adulteration in different types of meat and meat products. The species-specific primer technique employed here is better than barcoding because it is much faster and the sequence data from cyt b genes are available on DNA databases for many species and cheaper. However, this assay has its limitations in that it cannot provide quantitative data, unlike real-time PCR (12), Thus, quantitative real-time PCR technique to quantify the presence of animal material in foodstuff samples was suggested especially for the identification of small amounts of DNA.

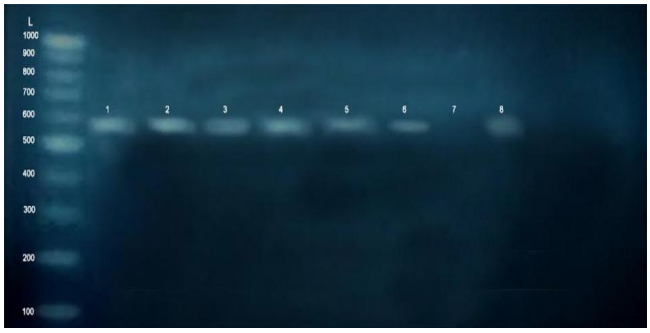

Figure 3: PCR product with 585 bp using sheep specific primer in 1.2% agarose gel electrophoresis, lane L; DNA ladder, lane 1; mixture contain 75% sheep meat, lane 2: 70% sheep meat, lane 3: 65% sheep meat, lane 4: 60% sheep meat, lane 5: 55% sheep meat, lane 6: 50% sheep meat, lane 7 and 8 were Negative and positive controls, respectively.

Conclusions

It was concluded that mitochondrial DNA (mtDNA) based PCR procedure used in this study proved to be effective and reliable in detecting the animal origin in kebab to level of 10% and that the processing cooking (grinding) and addition of non-meat ingredients showed no hindrance in the detection of meat species.

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