Identification of Some Meat Species Using PCR and Multiplex PCR of Mitochondrial Cytochrome B Gene

Abdul-Hassan, I.A.¹ and Tauma, J.A.²

¹ Genetic Engineering and Biotechnology Institute for Postgraduate Studies, Baghdad University.
² Ministry of Agriculture, Iraq.

Abstract

The objective of this study was the designing of a fast and reliable multiplex polymerase chain reaction (PCR) identification system for testing the pure species origin of meat samples. The present study involved the application of molecular biological approach to identify and differentiate some meat species i.e. cattle, buffalo, sheep, goat, chicken, horse and pig. The DNA was isolated from meat samples of each species and a part of the mitochondrial cytochrome b gene was amplified through PCR.

Different lengths of specific PCR products were detected to be 157,227,274,274,331,398 and 439 bp for goat, chicken, cattle, buffalo, sheep, pig and horse, respectively. Also, the identification of all meat species studied was determined by multiplex PCR using species-specific primers on mitochondrial cytochrome b gene. The results of the present study demonstrate that multiplex PCR is characterized by high efficiency and sensitivity even with very small amounts of fresh meat.

It is concluded that multiplex PCR can be useful for fast, easy, and reliable control of adulterated consumer meat products.

Introduction

In the last few decades, adulteration of food products has become a considerable problem in many countries as well as in Iraq. Consumers require clear and accurate information to make informed choices about their diet and the foods they buy. The information given to consumers is essential for them choosing one food product over another. Identification of the species origin in meat samples is relevant to consumers for several reasons: (1) possible economic loss from fraudulent substitutions or adulterations, (2) medical requirements of individuals who might have specific allergies, and (3) religious reasons (Miguel et al., 2004).

Developments in molecular biology have facilitated identification of plant, bacteria, and animal species with high accuracy (Sun and Lin, 2003; Tantillo et al.,

¹ Part of M. Sc. Thesis of the Second author
Recently, DNA-based methods have been considered as essential tools for species identification in animal foods and feedstuffs. In particular, the polymerase chain reaction (PCR) technique using species specific primers is extensively used because of its potential for simple, fast, specific and sensitive analysis, enabling the identification of species even in complex and processed foods (Mafra et al., 2008). Multiplex PCR, in which many primers were used together for amplification of multiple target regions, is a hopeful technique for meat species identification. Mitochondrial DNA (mtDNA) is a good target for phylogenic reconstruction at several taxonomic levels. Phylogenic approaches normally use sequences from a single gene such as Cyt b and COI, which are utilized for species and family level analysis (Hebert et al., 2004), as well as for resolution of taxonomic controversies. The increase in mutation rate of mtDNA is 5-10 times relative to a single copy nuclear gene which resulted in an accumulation of base substitutions over a long period of time. In authenticating food products, a number of mtDNA genes are used as target for detecting or isolating different animal species. Cytochrome b gene region is one of the conserved regions used as a molecular marker for this purpose (Hsieh et al., 2001). Cytochrome b is a functional gene located between genes responsible to produce tRNA^Glu^ and tRNA^Thr^ which encode partial cytochrome c oxidoreductase, a complex enzyme in oxidative phosphorylation (Leonard and Schapira, 2000; Southern et al., 1988). This gene contains species specific information, and it has been widely used in a considerable number of studies on phylogenesis and in studies dealing with forensic science and food inspection (Zarringhabaei et al., 2011).

The present study is focused on the use of conventional PCR and multiplex PCR and the reliability of cytochrome b gene variability in rapid detection and identification of chicken, sheep, goat, cattle, buffalo, horse and pig meat species.

**Materials and Methods**

Fresh raw meat samples were purchased from local markets in Baghdad and transferred to the laboratory in a chilled condition using an ice container. The meat were cattle (*Bos Taurus*), buffalo (*Bubalus bubalis*), chicken (*Gallus gallus*), goat (*Capra hircus*), sheep (*Ovis aries*), pork (*Sus scrofa domesticus*). Meat samples of horse (*Equus caballus*) were collected by biopsy. All samples were stored at (-20°C) until use.

Meat tissue was cut into small pieces with a sterile scalpel and separated out any adherent adipose tissue from it. Then the meat samples were subjected to DNA extraction using Tissue mini kit (Qiagen, USA). Meat sample (25 mg) was incubated with 180 µl of ATL buffer with 20 µl proteinase K at 56°C overnight and then for 1 h with 200 µl buffer AL. Then vortex for 15 seconds, after that, 200 µl ethanol were added, then vortex for 15 seconds. The resulting mixture was applied to the DNeasy mini spin column. Then the mixture was centrifuged at 8000 rpm for 1 minute. Then,
discard flow-through and collection tube. Thereafter, place the DNeasy mini spin column in a new 2 ml collection tube. Then, 500 µl of buffer AW1 were added, then, centrifuge for 1 minute at 8000 rpm. Then, discard flow-through and collection tube. Thereafter, place the DNeasy mini spin column in a new 2 ml collection tube. Then, 500 µl of buffer W2 were added, then, centrifuge for 3 minute at 14000 rpm. Then, discard flow-through and collection tube. Finally, place the DNeasy mini spin column in a clean 1.5 ml micro centrifuge tube and 200 µl of buffer AE were directly added onto the DNeasy membrane. Incubate at room temperature for 1 minute. The DNA bound to the column was washed in two centrifugation steps (8000 rpm for 1 minute) using buffer AE to improve the purity of the eluted DNA. Purity and concentration of the extracted DNA were assessed by using nanodrop (Optizen).

Multiplex PCR primers for the amplification of goat, chicken, cattle, sheep, pig and horse meat were designed as described by Matsunaga et al. (1999). Species specific primers (Table 1) were synthesized at Cinagen. The primer sequences were derived from the cytochrome b gene sequences from various species.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer</th>
<th>Sequences (5’ – 3’)</th>
<th>No. of Bases</th>
</tr>
</thead>
<tbody>
<tr>
<td>common</td>
<td>F</td>
<td>5'-GACCTCCCAGCTCCATCAAACATCTCATCTTGTGATGAAA-3'</td>
<td>38bp</td>
</tr>
<tr>
<td>goat</td>
<td>R</td>
<td>5'-CTCGACAATTGTGAGTTACAGAGGGA-3'</td>
<td>26bp</td>
</tr>
<tr>
<td>chicken</td>
<td>R</td>
<td>5'-AAGATAACAGATGAAGAAGAATGAGGCG-3'</td>
<td>27bp</td>
</tr>
<tr>
<td>cattle</td>
<td>R</td>
<td>5'-CTAGAAAAATGTAAGACCCGTAATATAAG-3'</td>
<td>29bp</td>
</tr>
<tr>
<td>sheep</td>
<td>R</td>
<td>5'-CTGAGATGCTAGTGGGCTATTTGTGCA-3'</td>
<td>26bp</td>
</tr>
<tr>
<td>pig</td>
<td>R</td>
<td>5'-GCTGATAGTAGTTTGTGATGACCGTA-3'</td>
<td>27bp</td>
</tr>
<tr>
<td>horse</td>
<td>R</td>
<td>5'-TCAGATTCACTCGACGAGGCTAGTA-3'</td>
<td>28bp</td>
</tr>
</tbody>
</table>

The 25-µl reaction mixture was prepared in an Eppendorf tube containing 12.5 µl of master mix, 8.5 µl of RNase-free water, 1 µl of 10X primer forward, 1 µl of 10X primer reverse and 2 µl of target DNA. The thermocycler was programmed for 35-cycle PCR. PCR was optimized with different annealing temperatures. The optimal annealing temperature was 62 °C for all primers. Each cycle included holding at 94 °C for 30 s, at 62 °C for 30 s, and at 72 °C for 30 s. Electrophoresis was run on agarose gel (1.5%) at 50 V for 1 h on a 10-µl portion of the amplified DNA fragments. The resulting gel was stained with ethidium bromide (0.5 µg/ml), visualized using a UV transilluminator, and photographed with a Polaroid 322 camera. The experiments were conducted in triplicate.
The 50-μl reaction mixture was prepared in an Eppendorf tube containing 25 μl of master mix, 7 μl of RNase-free water, 15 μl of 10X primer mix, and 3 μl of target DNA (mix all species). The thermocycler was programmed for 35-cycle PCR. PCR was optimized with different annealing temperatures. The optimal annealing temperature was 62 °C for all primers. Each cycle included holding at 94 °C for 30 s, at 62 °C for 90 s, and at 72 °C for 90 s. Electrophoresis was run on agarose gel (1.5%) at 100 V for 40 minute on a 12-μl portion of the amplified DNA fragments. The resulting gel was stained with ethidium bromide (0.5 μg/ml), visualized using a UV transilluminator, and photographed with a Polaroid 322 camera. The experiments were conducted in triplicate.

**Results and Discussion**

Development of simple and authentic method for detecting the species origin of a wide variety of meat continues to be a major challenge before the meat analysis (Rajni kumar, 2007).

Nucleic acid based techniques; popularly known as molecular techniques involve the DNA analysis. Analysis of DNA, rather than protein has been exploited for species identification due to its stability at high temperatures and its conserved structure within all tissues of an individual. DNA based techniques have been further simplified and benefited from introduction of PCR (Fairbrother et al., 1998).

The purity of the extracts obtained from all meat samples was high, since the ratio A260/A280 range between 1.7 and 2.0. Also, the DNA yields ranged between 1.6 and 2.0 μg/μl.

The PCR technique is highly susceptible to contamination due to its high sensitivity. It is very difficult to determine the source of contamination, because theoretically a single copy of template could be amplify after one PCR amplification assay. Moreover, PCR amplifies target sequence 10⁹ fold, producing enough DNA to serve as template for the next PCR reaction. Each amplicon can generate another positive result (Lei et al., 2008).

Several analytical approaches have been made to identify animal species in food products in order to protect consumers from fraud and adulteration such as polymerase chain reaction (Olivier Fumiere et al., 2006). Molecular methods for species detection using DNA are widely applied in food diagnostics (Dietrich Maede, 2006). A number of PCR-based methods for specialized in meat and meat products have been developed in the last few years (Colgan et al., 2001). The polymerase chain reaction (PCR) is a simple way to quickly amplify specific sequences of target DNA from indicator organisms to an amount that can be viewed by the human eye with a variety of detection devices (Amanda Fairchild et al., 2006).

Use of cyt b gene amplification for meat species identification offers a
promising strategy as cyt b sequences are good tools for studying phylogenetics of closely related species. The variable regions of the cyt b gene offer two main advantages: first that mt DNA is present in thousands of copies per cell (as many as 2,500 copies), especially in the case of post - mitotic tissues such as skeletal muscle (Greenwood and Paboo, 1999). This increase the probability of achieving a positive result even in the case of samples suffering severe DNA fragmentation due to intense processing conditions (Bellagamba et al., 2001) and the second large variability of mtDNA targets as compared with nuclear sequences facilitates the discrimination of closely related animal species even in the case of mixture of species (Hopwood et al., 1999 and Prado et al., 2002).

In this study, the cyt b gene fragments were amplified from DNAs of meat samples using a pair of primers, the forward and the reverse primers which were species specific by conventional PCR to identify meat species (goat, chicken, cattle, buffalo, sheep, pig, horse).

A forward primer was designed on a conserved DNA sequence in the mt cyt b gene, and reverse primers on species-specific DNA sequences for each species (Anderson et al., 1982; Desjardins and Morais, 1991 and Irwin et al., 1991).

In the preliminary phase of this study, conventional PCR Assays were carried out for individual identification of meat species. For each of the PCR, amplicons of expected size was obtained which was confirmed by running parallel a 100 bp marker (Figure 1). The objective of this figure was to confirm successful amplification of cytochrome b gene fragment in all meat species under study and to confirm the product size in various species. All the independent PCR, amplified the fragments of expected size, i.e. 157, 227, 274, 331, 398 and 439 bp for goat, chicken, cattle, sheep, pig and horse, respectively. When figure (1) was repeated again but cattle was replaced by buffalo, buffalo specific PCR produced 274 bp amplicon which identical to that of cattle. Cattle reverse primer was used to amplify cyt b gene fragment in buffalo.

In addition to the advantages of traditional PCR, such as high speed, good detection limit, selectivity, specificity and sensitivity; multiplex PCR allows the detection of multiple targets at the same time. Multiplex PCR allows the simultaneous amplification of more than one target sequence in a single reaction, multiplex PCR is slowly becoming adopted in both research laboratories and routine testing.

In this study, mitochondrial cytochrome b gene was amplified by multiplex PCR using a common forward primer and species specific reverse primer. This study also show that designed primers (Matsunaga et al., 1999) worked well in multiplex manner (Figure 2).

A multiplex PCR was designed by mixing all primers in a single reaction but targeting DNA of single species or mixed. Multiplex PCR was carried by mixing of primer in the different ratio viz., 1:0.1:0.1:0.1:0.2:0.1:0.1 for common forward: goat: chicken: sheep: cattle: pig: horse. Similar results were obtained by Matsunaga et al. (1999) for all the same species except buffalo. The PCR products showed species-specific DNA fragments of 157, 227, 274, 331, 398, 439 bps from goat, chicken, cattle, sheep, pig and horse, respectively. The multiplex PCR could detect up to ≤1µg of DNA of meat species.
Figure 1: PCR product sizes of mitochondrial \textit{cyt b} gene for the investigated species. lane1: chicken (227bp); lane2: cattle (274bp); lane3: sheep (331bp), lane4: goat (157bp); lane 5: pig (398bp); lane 6: horse (439bp); M 100: 100bp ladder.

Figure 2: multiplex PCR on six meat species. Goat (157bp); Chicken (227bp); cattle (274bp); sheep (331bp); pig (398bp) and horse (439bp) targeting \textit{cyt b} gene. M100: 100 bp ladder.

Compared to single-species PCR systems, multiplex PCR, in which many primers are used together for the amplification of more than one target region, is a hopeful technique to save costs and enhance the speed, efficiency and reliability of analysis for the simultaneous identification various meat species (Tobe and Linacre,
Moreover, multiplex PCR in this study could not differentiate cattle meat from buffalo meat. The results obtained in this study demonstrate the suitability of multiplex PCR analysis of the cyt b gene to identify cattle, sheep, goat, buffalo, chicken, pig and horse meat with the use of species specific primers.

Species identification of meat is important because of health, ethical and economic reasons (Wintero et al., 1990). The risk and thread of food adulteration and mislabeling have become a large concern and challenge for the food control sectors and consumers. Therefore, to enhance food security, fast and reliable detection methods are essential for the food industry. In order to enable food control authorities to supervise compliance with labeling requirements, suitable detection methods, which could allow unambiguous identification of animal or fish in a foodstuff are prudent (Zarringhabaie et al., 2011).

Using multiplex PCR, many targets are simultaneously amplified which helps in detection of many species in a short period of time. Also, multiplex PCRs must be developed with careful consideration for the regions to be amplified, the relative sizes of fragments, the dynamics of the primers and the optimization of PCR technique to accommodate multiple fragments (Edwards and Gibbs, 1995). Moreover, Fei et al. (1996) designed multiplex PCR primers based on mt D-loop DNA sequences and identify cattle, pig and chicken meats.

References


تشخيص بعض أنواع اللحوم باستخدام تفاعل السلسلة التبلري (multiplex PCR) لجين السايتوكروم-ب المايتوكونديري

الهدف من هذه الدراسة هو تصميم نظام تشخيص أنواع اللحوم باستخدام تفاعل السلسلة التبلري المتعدد (multiplex PCR) لتحديد وتمييز بعض أنواع اللحوم مثل: البازر، البايز، الإبل، الجاموس، الدجاج، الحنوان، الحنوان.

تم عزل الدنا من عينات اللحوم لكل نوع وتمت مصادفة جزء من جين السايتوكروم-B المايتوكونديري باستخدام تفاعل السلسلة التبلري.

تم الحصول على أطوال مختلفة من نواتج تفاعل السلسلة التبلري وهي 157، 227، 274، 331، 398 و 439 زوج قائمة للمؤخر، البازر، الجاموس، الإبل، البقر، الحنوان، الحنوان.

تعد هذه الدراسة أن تفاعل السلسلة التبلري المحدد يمتاز بكفاءة وحساسية عالية في حالة استعمال كميات قليلة جدا من اللحوم.

تستنتج من نتائج هذه الدراسة أن تفاعل السلسلة التبلري المحدد يمكن أن يكون فحص مفيد، سريع، سهل وفعال عليه في حالة تطبيقه لفحص منتجات اللحوم المغشوشة.

*جزء من رسالة ماجستير للباحث الثاني

Abdul-Hassan and Tauma


