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Polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) for identification of meat species and some meat products at Assiut City

by

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ABSTRACT

In this study, a polymerase chain reaction and restriction fragment length polymorphism analysis (PCR-RFLP)-based method was applied to identify the meat origin of different animal species by using a universal primer cytochrome b (*cyt b1* and *cyt b2*). It is common in to adulterate buffalo's meat for sale to consumers. Identification of the species of origin for meat and products is important and useful in practice to protect human from adulteration, because it allows the detection of fraud in the form of the substitution of a less costly type of meat for one of a higher quality. Meat samples were collected from 4 farm animals' species (cattle, buffalo, goat and sheep) to differentiate each species according to its mitochondrial DNA (mtDNA). This method was based on mtDNA conserved region sequence variations. The DNA sequence of mitochondrial cytochrome b gene is 359-bp was obtained from gene-bank data base (www.ncbi.nlm.nih.gov). Then the PCR product was digested, using restriction endonuclease enzymes and yield species-specific restriction profile. This technique is more sensitive and also specific for meat species identification and differentiation. Therefore, this assay may be suitable test and more rapid than conventional methods. The economic impact of this situation leads to many investigations of potential fraud meat and meat products. The mitochondrial encoded *cytb* gene was used as a molecular marker for the discrimination of meat and meat products species,

KEY WORDS

Meat, Meat products, PCR, RFLP analysis, mitDNA, Animal species.

INTRODUCTION

Several animal species such as cattle, buffalo, goat and sheep, provide meat and meat byproducts. Meat products represent a large proportion of the food industry. In many European countries, laws require producers to state the type of meat used for manufacturing. Illegal adulteration of raw materials to be used for the commercial preparation of food is a common problem. This type of adulteration should be detected by adequate control methods to check for the presence of meat type in such dairy products. PCR is a simple procedure that was developed to identify the species origin of meat used for meat byproducts. During the last few years, many studies have demonstrated the utility of PCR for the detection of a very small amount of DNA molecules **(Taberlet *et al.*, 1996)**. In particular, PCR has a high potential because of its increased sensitivity and specificity when compared with protein assays and hybridization with DNA probes (Meyer and Candrian, 1996). PCR was used to amplify homologous segments of mitochondrial DNA (mtDNA) from more than 100 animal species, including mammals, birds, amphibians, fish and some invertebrates. Universal primers are directed towards the conserved regions of *cytb* gene of various animal species **(Kocher et al., 1989).** According to **Lockley and Bardsley (2000)**, PCR assays allow discrimination of closely related animal breeds. DNA-probes have been developed for the identification of cattle, sheep, goat, horse, deer, pig, chicken and turkey species **(Chikuni et al., 1990)**, and were also used in determining species of raw meat as well as heat processed meat **(Baur et al., 1989)**. **Tartaglia et al. (1998)** reported that the PCR-RFLP analysis is a specific, rapid, simple and highly sensitive technique, which is successfully used to detect and identify bovine-derived meats and bone meals in ruminant feeds. This method detects the presence of the bovine mtDNA in feedstuffs at level less than 0.125%. **Lenstra et al. (2001)** recorded that PCR-RFLP is the most suitable technique for species identification of

meat products, especially in case of degraded samples and also for the detection of admixture, the technique, however, is sensitive to contamination.

MATERIALS & METHODS

Collection of meat and some of meat products samples:

From the slaughter house, Assiut, Egypt, meat samples and some meat products were collected in sterile plastic bags from 4 different farm animals species, cattle (*Bos taurus*), buffalo (*Bubalus bubalis*), goat (*Capra hircus*) and sheep (*Ovis aries*). These animals' species are consisting 4 genuses and one family Bovidae. The samples were kept frozen in a freezer at –20˚C until use for DNA extraction.

DNA extraction:

Genomic DNA from meat and meat byproducts samples were extracted according to **Koh et al. (1998)**. Meat samples 100 mg were digested with 250 µl SSE buffer (0.3 M Sodium acetate, 1% SDS, and 5 mM EDTA, pH 8.3) and 50 µl proteinase K. The mixture was incubated at at 56 ˚C in a water bath for overnight, and then centrifuged at 13.000 xg for 3 minutes. The supernatant containing DNA was transferred to clean sterile tube and subjected to purification with Qiagen tissue kit. Then the tube contain DNA stored at -20˚C until use for PCR.

DNA amplification:

Amplifications of DNA for *cytb* PCR were carried out in 25 µl reaction volumes containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM each of the four dNTP′ s (dATP, dCTP, dGTP, dTTP), 1 unit of Ampli Taq Gold® polymerase (Applied Biosystems, GmbH, Darmstadt, Germany), 5 µl DNA and 0.5 µM of each of the oligonucleotide primers. The universal primers used were shown to be complementary to the conserved regions of the mitochondrial cytochrome b gene in vertebrates. The primer sequences used were as follows **(Kocher *et al.*, 1989)**:

Cytochrome b 1 (*cytb 1*): 5′-CCATCCAACATCTCAGCATGATGAAA-3′ Forward primer

Cytochrome b 2 (*cytb 2*): 5′-GCCCCTCAGAATGATATTTGTCCTCA-3′ Reverse primer

The reaction mixture was overlaid with one drop of mineral oil and centrifuged at 13.000 xg for 15 seconds. The tubes were transferred immediately into the thermal cycler.

Cycling parameters:

The polymerase chain reaction was carried out in a thermocycler programmed to perform a denaturation step at 95ºC for 10 minutes (to activate the Taq Gold® DNA Polymerase), followed by 35 cycles consisting of 45 seconds at 94ºC, 30 seconds at 52ºC and

45 seconds at 72ºC. The last extension step was 5 minutes longer. The samples were stored at 4ºC until use in the next step.

Gel electrophoresis:

Two percent agarose gel containing 2.5 µl ethidium bromide in 1x electrophoresis buffer was prepared. The electrophoresis chamber was filled with buffer solution (1x electrophoresis buffer) until the top surface of the gel submerged by approximately 1 mm (about 400 ml). Two microlitres of the sample buffer was mixed with 10 µl PCR product and centrifuged at 13.000 g for 20 seconds. The DNA ladder (100 bp) was prepared. The samples (10 µl) and DNA ladder (10 µl) were placed into the agarose gel wells. The chamber was connected to a 60 Volt power supply and the run was initiated. The separated DNA products were detected using UV transillumination. The PCR products containing a DNA sequence of 359 bp were amplified and later photographed using a Polaroid® camera.

DNA sequences:

The mitochondrial cytochrome b (*cytb*) gene sequences (359 bp fragments) of the respective vertebrates were obtained from the available home page of the National Center of Biotechnology Information (NCBI):
(http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html)
The fragment sequences animal species are listed in Figure 1.

obtained from the gene bank database for 4 animal species.

```
     Cytochrome b1                                                          HaeIII
1    ccatcaaacatttcatcatgatgaaatttcggttccctcctgggaatctgcctaatcctacaaatcctcacaggc Cattle
1    ccatcaaacatctcatcatgatgaaactttggctctctcctaggcatctgcctaattctgcaaatcctcaccggc W. Buffalo
1    ccatcaaacatctcatcatgatgaaactttggatccctcctaggaatttgcctaatcttacaaatcctgacaggc Goat
1    ccatcaaatatttcatcatgatgaaactttggctctctcctaggcatttgcttaatttacagattctaacaggc Sheep

76   ctattcctagcaatacactacacatccgacacaacaacagcattctcctctgttacccatatctgccgagacgtg Cattle
76   ctattcctagcaatacactacacatccgacacaacaacagcattctcctccgtcgcccacatctgccgagacgtg W.Buffalo
76   ctattcctagcaatacactatacatccgacacaataacagcattttcctctgtaactcacatttgtcgagatgta Goat
76   ctattcctagcaatacactatacacctgacacaacaacagcattctcctctgtaacccacatttgccgagacgta Sheep
                    TaqI                AluI
151 aactacggctgaatcatccgatacatacacgcaaacggagcttcaatggtttttatctgcttatatatgcacgta Cattle
151 aactatggatgaattattcgatacatacacgcaaacggagcttcaatatttttcatctgcttatatatacacgta W. Buffalo
151 aattatggctgaatcatccgatacatacacgcaaacggagcatcaatattctttatctgcctattcatacatatc Goat
151 aactatggctgaattatccgatatatacacgcaaacggggcatcaatatttttatctgcctatttatgcatgta Sheep


226 ggacgaggcttatattacgggtcttacacttttctagaaacatgaaatattggagtaatccttctgctcacagta Cattle
226 ggacgaggcatatactacggatcatatacctttctagaaacatgaaacatcggagtaatttctattattcgcagta W. Buffalo
226 ggacgaggtctatattatggatcatatacctttctagaaacatgaaacattggagtaatcctcctgctcgcaaca Goat
226 ggacgaggcctatactatggatcatatacctttcctagaaacatgaaacatcggagtaatcctcctatttgcgaca Sheep
                                  Cytochrome b2
301 atagccacagcatttataggatacgtcctaccatgaggacaaatatcattctgaggagc Cattle acces No. AF490529
301 atagccacagcatttataggatacgtactgccatgaggacaaatatcattctgaggggc W.Buffalo acc. No.D82892
301 atggccacagcattcataggctatgttttaccatgaggacaaatatcattttgaggggc Goat acc.no. AB004075
301 atagccacagcattcataggctatgttttaccatgaggacaaatatcattctgaggagc Sheep acc. No.010406
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Figure 1. DNA sequences from part of the mitochondtial Cytb gene (359 Bp) of different animal species aligned together.

Restriction fragment length polymorphism (RFLP) analysis:

For RFLP analysis of the *cytb* products, restriction endonucleases were selected from restriction maps of *cytb* sequences of the animal species of the family Bovidae. The selection criteria were based on the minimum number of enzymes required to produce diagnostic restriction fragment profiles. To satisfy these criteria, three enzymes, *Taq* I, *Alu* I and *Hae*III, which recognize the sequences T↓CGA, AG↓CT and GG↓CC respectively, were selected. Sites for restriction enzymes that cleave a 359 bp-fragment were identified by means of the program *NEB cutterVI.0* designed by New England Biolab Incorporation (NEB Inc.): http://tools.neb.com/NEBcutter/.

RESULTS and DISCUSSION

DNA sequences of different animal species:

Figure 1 shows part of the aligned nucleotide sequence of the *cytb* gene that has been determined for 4 animal species (Cattle, Buffalo, Goat and sheep). The differences in the *cytb* sequence in closely related species would help in inter-species differentiation, using restriction enzymes (*Taq*I, *Alu*I and *Hae*III).

Amplification of mtDNA from meat of different animal species:

In the present study, it was possible to amplify specific PCR products from DNA extracted from various meat and meat products was successfully amplified using the universal cytochrome b primer (*cytb 1* and *cytb 2*). A single PCR amplicon, corresponding in size to the predicted 359 bp fragment was observed **(Figure 2)**.

Identification of animal species by PCR-RFLP analysis:

*Taq*I restriction sites were found in some tested animal species **(Figure 3)**. All resultant fragment-sizes with *Taq*I were in conformity with the expected sizes according to the gene bank records. The *Taq*I fragments of the *Capra hircus* (goat) amplicons were 218 bp and 141 bp while the *Bubalus bubalis* (water buffalo) amplicons were 168 bp and 191 bp. No *Taq*I restriction sites were found in fragments 359 bp amplified from *Bos taurus* (cattle) and *Ovis aries* (sheep). *Alu*I restriction fragments of the cattle and water buffalo amplicons were 190 bp and 169 bp **(Figure 4)**. No *Alu*I restriction sites were found in fragments amplified from DNA of goat and sheep; this was in conformity with the expected sizes

according to the gene bank records. The *cytb* amplicons of the 4 animals species were also analyzed with *Hae*III **(Figure 5)**. *Hae*III restriction analysis of cattle and water buffalo amplicons resulted in the expected fragments of 285 bp and 74 bp. *Hae*III fragments of the goat amplicons resulted in 230 bp, 74 bp and 55 bp fragments. Sheep amplicons restriction fragments 285 bp, 159 bp, 126 bp and 74 bp were not the same as the expected sizes in the gene bank records. **Partis *et al.* (2000)** reported that difference between results and expected sizes from gene bank records might be attributed to co-amplification of nuclear *cytb* pseudogenes. PCR-RFLP is a promising method for the determination of both cooked and uncooked tissues, Moreover, the technique provides a simple, rapid and less expensive method, alternative to sequencing of PCR products, and allows the discrimination of species, even when the template DNA may be degraded or contains only traces of DNA **(Lenstra et al., 2001).**

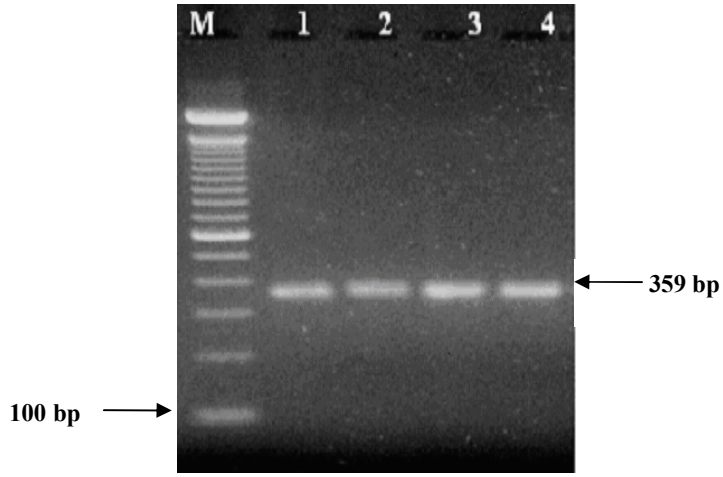

Figure 2. PCR Products in different animal species (359 bp), M 100 pb Molecular DNA marker, 1, cattle DNA, 2. Buffalo DNA, 3, Goat DNA, 4, Sheep DNA

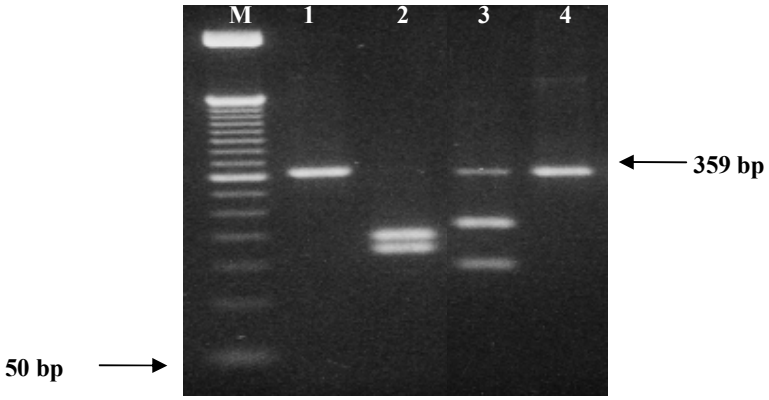

Figure 3. Restriction profiles of cytochrome b PCR amplicons digested with TaqI, M, 50 bp molecular marker, Lanes 1-4, DNA samples from meat and meat products, cattle, buffalo, goat and sheep, respectively.

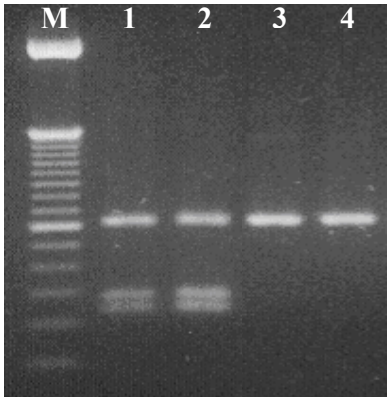

Figure 4. Restriction profiles of cytochrome b PCR amplicons digested with AluI, M, 50 bp molecular marker, Lanes 1-4, DNA samples from meat and meat products, cattle, buffalo, goat and sheep, respectively.

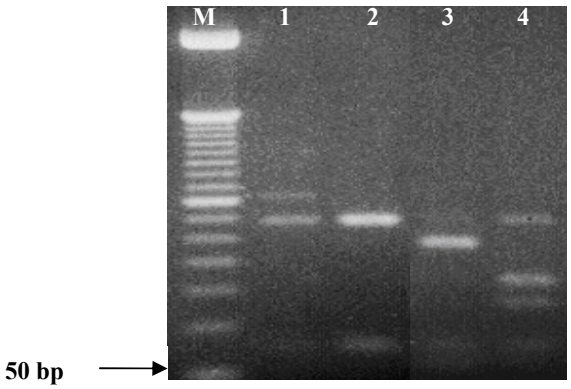

Figure 5. Restriction profiles of cytochrome b PCR amplicons digested with HaeIII, M, 50 bp molecular marker, Lanes 1-4, DNA samples from meat and meat products, cattle, buffalo, goat and sheep, respectively.

Evaluation of the PCR-RFLP approach revealed that it is a simple and reliable technique for meat species identification. Three different restriction enzymes (*Taq*I, *Alu*I and *Hae*III) were found to be sufficient for the discrimination of 3 investigated animal species of the family Bovidae.

The method used in this study, shows promise for the identification of both cooked and uncooked meats. In addition, it can be used to identify the species of autoclaved (120ºC) mixed meat samples to levels of less than 1% **(Sun and Lin, 2003)**. Moreover, **Partis and co-authors (2000)** reported that the method can be used for the identification of almost all vertebrate species.

REFERENCES

Baur, C.; Teifel-Greding, J. and Liebhardt, E. (1989):
Species identification by fragmented DNA.
Beitr Gerichtl Med, 47, 165-170.

Chikuni, K.; Ozutsumi, K.; Koishikawa, T. and Kato, S. (1990):
Species identification of cooked meats by DNA hybridization assay.
Meat Sci, 27, 119-128.

Chikuni, K.; Tabata, T.; Kosugiyama, M. ; Monma, M. and Saito, M.(1994):
Polymerase chain reaction assay for detection of sheep and goat meats.
Meat Sci, 37, 337-345.

Ebbehoj, K.F. and Thomsen, P.D. (1991):
Differentiation of closely related species by DNA hybridization.
Meat Sci, 30, 359-366.

Kocher, T.D.; Thomas, W.K.; Meyer, A.; Edwards, S.V.; Pääbo, S.; Villablanca, F.X. and Wilson, A.C. (1989):
Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers.
Proc Natl Acad Sci USA, 86, 6196-6200.

Lenstra, J.A.; Buntjer, J.B. and Janssen, F.W. (2001):
On the origin of meat - DNA techniques for species identification in meat products.
Vet Sci Tomorrow, 2, 1-13.

Lockley, A.K. and Bardsley, R.G. (2000):
Novel method for the discrimination of tuna (*Thunnus thynnus*) and bonito (*Sarda sarda*) DNA.
J Agric Food Chem, 48 (10), 4463-4468.

Matsunaga, T.; Chikuni, K.; Tanabe, R.; Muroya, S.; Shibata, K.; Yamada, J. and Shinmura, Y. (1999):
A quick and simple method for the identification of meat species and meat products by PCR assay.
Meat Sci, 51, 143-148.

Meyer, R.; Candrian, U. and Lüthy, J. (1994):

Detection of pork in heated meat products by polymerase chain reaction (PCR).
J AOAC Int, 77 (3), 617-622.

Partis, L.; Croan, D.; Guo, Z.; Clark, R.; Coldham, T. and Murby, J. (2000):
Evaluation of a DNA fingerprinting method for determinating the species origin of meats.
Meat Sci, 54 (4), 369-376.

Sun, Y.L. and Lin, C.S. (2003):
Establishment and application of a fluorescent polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for identifying porcine, caprine and bovine meats.
J Agric Food Chem, 51 (7), 1771-1776.

Tartaglia, M.; Saulle, E.; Pestalozza, S.; Morelli, L.; Antonucci, G. and Battaglia,
P.A. (1998):
Detection of bovine mitochondrial DNA in ruminant feeds: A molecular approach test for the presence of bovine-derived materials.
J Food Prot, 61 (5), 513-518.

اختبار تفاعل البلمرة والتحليل الجزيئى متعدد الاوجه للتعرف على انواع اللحوم وبعض منتجاتها بمدينة اسيوط

احمد عبدالراضى و لبنى محمد

الملخص العربى

فى هذه الدراسة تم تطبيق اختبار تفاعل البلمرة والتحليل الجزيئى متعدد الاوجه فى التعرف والتفرقة بين لحوم الحيوانات من الفصائل المختلفة وذلك باستخدام يونيفرسال بادئ وهو عبارة عن سيتوكروم ب1 وب2. والشائع هو غش اللحوم ومنتجاتها وبيع الردئ منها على انه عالى الجودة او بيع لحوم لفصائل حيوانات ليست هى وذلك من اجل الربح الكثير والسريع. وكل هذا ينعكس على المستهلك وصحتة. ومن ثم التعرف على انواع مصادر اللحوم ومنتجاتها من فصائل الحيوانات المختلفة يعتبر مهم ومفيد للانسان. وخلال هذه الدراسة تم اخذ لحوم م فصائل حيوانات سائدة الاستخدام فى مصر مثل الابقار , الجاموس , الماعز والاغنام. وتم التعرف والتفرقة بينهم بواسطة الاختبار المستخدم فى التجربة. تم الاعتماد على تضاعف منطقة محافظة من السيتوكروم ب جين وهو موجود فى الميتوكوندريل DNA والمنطقة التى تم مضاعفتها هى 359 وهذا الجزء تم تقطيعه باستخدام انزيمات التقطيع وبالتالى ادى ذلك الى الحصول على شكل ثابت لكل فصيلة بعد استخدام الانزيم الخاص بهذه الفصائل من الحيوانات. وثبت من استخدام هذا الاختبار بانه ذو حساسية عالية وايضا نوعى يمكنه من التعرف والتفرقة بين الفصائل المختلفة. ومن ثم اعتبر الاختبار بانه ملائم واسرع من الاختبارات التقليدية التى تستخدم لهذا الغرض.