Application of PCR-RFLP of cytochrome *b* gene and species-specific PCR in differentiation between different kinds of meat

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Abstract :
DNA was extracted from cattle's sheep's, goat's, donkey's, pig's and dog's muscles and PCR-RFLP analysis was applied to identify the meat origin of the different animal species by using a universal primer of cytochrome b gene (cyt b1 and cyt b2). The PCR amplification product size in all animals muscles was 359 bp. Then the PCR product was digested, using restriction endonuclease enzymes, Alu I, Hinf I and Hae III, and yield species-specific restriction profile. Moreover,The specific polymerase chain reaction (PCR) technique was used for detection, identification and authentication in raw meat samples by using species-specific primers. The PCR amplification product size in cattle's, sheep's, goat's, donkey's, pig's and dog's meat were 271, 225, 157, 439, 212 and 322 bp, respectively. The results showed that cytocrome b gene PCR-RFLP analysis and species-specific PCR provide a rapid and effective methods to detect and differentiate between the meat species. But, cytocrome b gene PCR-RFLP cannot defined a composite profile from a sample of mixed origin and may not reflect quantitatively its species composition. The species-specific PCR methods are highly sensitive and will improve the detection limits for DNA sequences derived from mixed meat of different species.
Keywords: Meat, Meat products, PCR, RFLP analysis, mitDNA, Animal species.

Introduction

Food authenticity is currently an issue of major concern for food authorities, since incorrect labeling of animal foods may have remarkable negative consequences.

The application of quality assurance systems through the food chain requires the development of reliable and simple tools, which facilitate routine control assessments. The detection of meat species in different foods and feedstuffs deserve special attention due to the recent crisis in the meat sector (Brodmann and Moor, 2003). On other hand, incorrect labeling represents commercial fraud as regards the consumer, in particular when this involves replacement of animal species by another of lower commercial value. On the other hand, incorrect labeling may also have implications for health, especially in case of consumers who exhibit sensitivity to undeclared antigens. Those following religious dietary restrictions are also defrauded with such replacement like Hindu population also choose not to eat beef, while Jewish and Muslim populations choose to avoid consumption of pork, even in minute quantities, due to their religious beliefs and also for the conservation of endangered species.

Bovine spongiform encephalopathy (BSE), also known as mad cow disease, has been detected in 26 countries including Canada and the United States. Consumption

of meat from BSE-infected cattle is believed to have caused the death of ne 200 people worldwide, from a disease called variant Creutzfeldt-Jakob dise (vCJD) (GAO, 2005). It is widely believed that the practice of utilizing rumi carcasses in animal feed for livestock is responsible for the spread of BS epidemic proportions. As a result, the need for sensitive detection of rumi species remains in animal feed is a paramount agricultural issue (Corona, 2007).

Methods used for identification of species of origin of raw meat incl sensory analysis, anatomical differences, histological differentiation of the that may possibly exist in the meat, properties of tissue fat, and level of glyco in muscle tissue, as well as electrophoresis and DNA hybridization (Saez al., 2004). Most of these methods have been reported to have limitations in due to problems in specificity (i.e. sensory analysis, glycogen level, histolog differentiation, properties of tissue fat, and immunological methods), comple (i.e. electrophoresis and DNA hybridization), high cost (i.e. DNA hybridizati and some requirements for baseline data about the differences in pro compositions (i.e. isoelectrofocusing) (Matsunaga et al.,1999 and Meyer et 1995). There is a need for the development of a more accurate, fast, and ea to-use method due to the limitations of the existing methods mentioned ab (Matsunaga et al.,1999).

Mitochondrial DNA (mtDNA) accumulates about 10 times as m mutations per unit as nuclear DNA and has thousands of copies per cell. Th amplification of a mitochondrial DNA segment is a relatively sensitive proced and the identification of the species can be based on mutations in amplification products. A simple and convenient way of testing for a mutatio RFLP (Restriction Fragment Length Polymorphism) analysis, which uses enzyme with a recognition sequence created or abolished by the mutat Species identification using PCR-RFLP of a mitochondrial cytochrome b g (mt cyt b gene) has been well documented by Partiset al 2000. The techniqu equally applicable to the identification of species origin in cheese produ (Branciari et al 2000) as it is in meat products.

An alternative DNA detection system is based on the specific polymer chain reaction (specific-PCR) and the amplification of a segment of mitochondrial DNA specific for each animals species by using specific primer each animals meat species (Alhak and Arslan, 2007).

The present study was designed to investigate PCR methods identification and authentication for different kind of cattle, sheep, goat, donk dog and pig meat based on PCR-RFLP technique of cytochrome b ge moreover, uses of species specific PCR to identified pork , cattle, sheep, g donkey ,dog and pig meat separately and in meat mixture.

Materials and Methods

Meat samples: Meat samples of cattle, sheep, goat, donkey, dog and pig w purchased and collected from Cairo City, Egypt. DNA was extracted from e meat sample and stored at –20 ± 1 °C until analyzed.

Test meat mixtures: Each meat samples of cattle, sheep and goat were mi with meat samples of pig, dog and monkey separately. Following mixing, a portion of each sample was taken separately from each test mixture. DNA v extracted from each test meat sample and used for PCR analysis.

DNA Extraction: DNA was extracted from each meat sample and each test meat sample by the DNeasy protocol provided with animal and Fungi DNA Preparation Kit (Jena Bioscience Cat. No. PP-208S). Extraction was performed on 5-10 mg of fresh or frozen meat sample on a 1.5 ml microtube. containing 300 µl Cell Lysis Solution and 1.5 µl Proteinase K (20 mg/ml). Incubate at 55°C overnight or until tissue has dissolved. At the second day add 100 µl of Protein Precipitation Solution to the cell lysate and mix well by vortexing. Then centrifuge at 15,000 g for 3 min. (The precipitated protein will be a tight pellet). DNA was precipitated from the supernatant by isopropanol alcohol 99% and washed by ethanol alcohol 80%. After the DNA was pelleted by centrifugation and air dried , the DNA was dissolved in 50 ul hydration solution containing 1.5 ul RNase A at 37 °C for 30 min. then at 65 °C for 60 min.(Mohamed, et al 2007).

Polymerase chain reaction

Oligonucleotide primers: Two set of primers were used specific for cytochrome b gene (Johannes,et al., 2001) . PCR primers for the amplification of bovine, sheep, goat, horse and pig meat were designed as described by Lahiff et al. (2001) and Matsunaga et al. (1999). Species specific primers for the detection of dog were designed as described by Alhak and Arslan, 2007. The sequence of the primers were illustrated in table(1).

	Sequences 5-3	Amplified products
Cyt. b gene (common)	5`-CCA TCC AAC ATC TCA GCA TGA TGA AA-3`	359 bp
	5`-GCC CCT CAG AAT GAT ATT TGT CCT CA-3`	
Bovine	5'- GCCATATACTCTCCTTGGTGACA- 3	271 bp
	5'- GTAGGCTTGGGAATAGTACGA- 3'	
Sheep	5'- TTAAAGACTGAGAGCATGATA- 3'	225 bp
	5'- ATGAAAGAGGCAAATAGATTTTCG- 3'	
Goat	5'- GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA- 3'	157 bp
	5'- CTCGACAAATGTGAGTTACAGAGGGA- 3'	
equine	5'- GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA- 3'	439 bp
	5'- CTCAGATTCACTCGACGAGGGTAGTA- 3'	
Porcine	5'- GCC TAA ATC TCC CCT CAA TGG TA- 3'	212 bp
	5'- ATG AAA GAG GCA AAT AGA TTT TCG- 3'	
Dog	5'- GAT GTG ATC CGA GAA GGC ACA- 3'	322 bp
	5'- TTG TAA TGA ATA AGG CTT GAA G- 3'	

Table 1: The sequences of the oligonucleotide primers used for the detection of cytochrome b gene as a universal primers and a panel of specific primers for cattle, sheep, goat, donkey, dog and pig meats.

DNA amplification: DNA amplification was done in 25 ul reaction vol containing 2.5 ul of 10X reaction buffer (65 mM Tris-Hcl, PH 8.8 at 25°C, 16 ammonium sulphate, 200 ug of gelatin per ml), 200 uM of each of deoxyribonucleotide triphosphates,10 pM of each oligonucleotide primer , 2 magnesium chloride, 1 U of Taq DNA polymerase and 50 ng of template I (Johannes,et al., 2001). PCR was carried out in a gene cycler (Perken E model 6900).

The optimized cycle program for PCR using cytochrome b gene primer denaturation, annealing and extension temperatures was as follow: i denaturation of 5 min at 94°C; 35 cycles of 1 minute at 94°C, 1 minute at 5 and 1min. at 72°C; and final extension step at 72°C for 5 min (Johannes,e 2001). After amplification a 5 ul of the reaction product was mixed with 1 ul c gel loading buffer and subjected to electrophoresis on 1.5% agarose gel at 1 for 30 min.. Gel were stained with ethiduim bromide and photographed or transilluminator (Ilhak and Arslan, 2007). Samples were considered pos when a single band of DNA at 359 bp were evident in the ethidium bro stained gels compared with the molecular size marker 100-bp DNA ladder (. Bioscience Cat. No. M-214). The gels were then photographed using a Pola Camera.

Digestion of the Amplified Products of cyt. b gene: H_2O was added to 1 ul PCR amplification product to obtain a final volume of 20 ul, 2 ul of appropriate 10 × enzyme reaction buffer and 5 U of restriction enzyme generate a specific pattern for each species, Alu I enzyme for cattle, donkey dog; Hinf I for cattle; and Hae III for sheep (Jena Bioscience Cat. No. EN-12 Incubate at 37°C for two hours, then analyze by electrophoresis on 2% aga gel (Johannes,et al., 2001).

For specific primers of cattle, sheep, goat, donkey, dog and pigs, specific polymerase chain reaction was optimized with different anne temperatures. The optimal annealing temperature was 50 °C for all prin Each cycle included holding at 94 °C for 1 min., at 50°C for 1 min. and at 7 for 1 min. , proceded with initial denaturation of 5 min at 94°C and followed final extension step at 72°C for 5 min.

Electrophoresis of PCR-RFLP Products: The PCR products loaded on ethidium bromide stained 2% agarose gel. Analysis of spe specific pattern were done by comparing the molecular weight of fragments with the reference DNA marker(100 bp DNA ladder Jena Biosci Cat. No. M-214 and 50 bp DNA ladder Jena Bioscience Cat. No. M 202) , photographed using a Polaroid Camera.

Results and Discussion

For discrimination of cattle, sheep, goat, donkey, dog and pig, PCR-F technique was used to amplify mt-DNA cytochrome *b* gene in above menti animals. As expected, the amplified fragment length of these species was bp (Fig. 1). The digestion of the amplified product of cytochrome *b* gene (35 using Alu I restriction enzyme to discriminate between cattle, donkey and yielded two fragments 190 bp and 169 bp only with cattle, 244 bp and 11 only with pig and four bands with donkey at 274,169, 105 and 85 bp, whe with other animals the amplified cytochrome *b* gene (359 bp) was not dige (Fig. 2), allowing an identification of cattle's meat in addition to pig and dor

By using Hinf I restriction enzyme, the digestion of cytochrome *b* gene (359 bp) of cattle yield three fragments at 198, 117 and 44 bp (Fig. 3). By using Hae III restriction enzyme , the digestion of the amplified product of cytochrome *b* gene of sheep yield three bands at 239, 74 and 55 bp, the digestion of the amplified product of cytochrome *b* gene of pig yield two bands at 153 and 74 bp and the digestion of the amplified product of cytochrome *b* gene of goat yield two bands at 239 and 74 bp (Fig. 3). The amplified fragment length of other species (359-bp) was not digested by Hinf I and Hae III enzymes.

Moreover, five set of species-specific primers were also used to amplify mitochondrial DNA (mt DNA) specific for cattle, sheep, goat, horse, dog and pigindependently. The PCR conditions were optimized on meat of each species separately. The PCR produced a single band of expected size at 271, 225, 157, 439, 322 and 212 bp in above mentioned animals meat respectively, confirming the species specificity of primers in PCR (Fig. 4). The primers used in this study are specific for its species and did not show any cross-reactivity with others.

By application of PCR by using pig specific primers on the test meat mixture, meat samples of cattle, sheep and goat which mixed with meat samples of pig, give a single band at 212 bp (Fig. 5), while , by application of PCR by using dog specific primers on the test meat mixture, meat samples of cattle, sheep, and goat which mixed with meat samples of dog give a single band at 322 bp. (Fig. 6).Also, by application of PCR by using horse specific primers on the test meat mixture, meat samples of cattle, sheep, and goat which mixed with meat samples of donkey give a single band at 439 bp.(Fig. 7). Results of the present study supported the findings published by Ilhak, and Arslan, (2007), Meyer et al. (1994,1995), Hopwood et al. (1999), and Partis et al. (2000), who reported that PCR could be used for identification of meat mixes at 0.5% levels.

PCR analysis of species-specific mtDNA sequences is the most common method currently used for species identification (Cann et al., 1987 and Parodi et al., 2002). Detection method based on mtDNA can improve the sensitivity further because each cell has only a set of genomic DNA in the nucleus, but bearing several copies of mtDNA which charecterized by high mutation rate, because they are located in cytoplasm and easier to be attacked. Since mtDNA expressed in different species or genuses have their evolution specificities, we can identify individual species by studying mtDNA. There are approximate 1000 mitochondria in a cell and 10 copies of mtDNA per mitochondrion, much copies of mtDNA are available per cell and just one copy for genomic DNA. Therefore, mtDNA efficiently to detect species-specific DNA than genomic DNA. The variation of mt cyt b gene has been a rich source of phylogenetic inference in a wide range of animal species. Thus, amplification of a mitochondrial DNA segment is a relatively sensitive procedure, and the identification of the species can be based on mutations in the amplification products. A simple and convenient way of testing for a mutation is RFLP analysis, which uses an enzyme with a recognition sequence created or abolished by the mutation. Species identification using PCR-RFLP of a mitochondrial cytochrome b segment has been well documented by (Partis et al., 2000). The technique is equally applicable to the identification of species origin in meat (Branciari et al., 2000 and Filipe, et al 2008).

Partis et al., 2000 suggested that the cyt b PCR-RFLP method was a promising one for the identification of both cooked and uncooked tissues, although the

method was unsuitable for analyzing meat mixtures. Johannes, et al. 2 mentioned that the primers of the cytochrome b fragment have been desig on the basis of the human sequence and have mismatches with homolo sequences. As a result, amplification with one species may be more effi than with another and a composite profile from a sample of mixed origin ma reflect quantitatively its species composition.

Modern molecular techniques, based on DNA analysis, have g applicability in detecting adulteration, and they represent useful complemen methods relying on protein analysis for the identification animal species. D based techniques have become effective and reliable for commercial products also (Feligini et al., 2005). The advantages of DNA-based analysis manifold. First is the ubiquity of DNA: that from all cell type of an indiv contains identical genetic information. Secondly, the information content of is more abundant compared to proteins due to the degeneracy of the ge codes. Thirdly, DNA is a rather stable molecule which renders DNA extra and analysis from many sample types feasible (Cheng et al., 2003).

Conclusion

In conclusion Nucleic acid based analysis has been widely used in many fi and has become increasingly popular for the differentiation and identificatio feed or food adulterants. The PCR assays described here might be usefu effective control of adulterated consumer meat products and violation labeling requirements for meat products. It is also a valuable tool for assessment of risk associated with introduction of animal material that migh harmful to human or animal health. They are rapid, simple and applicabl detection The meat species in different foods and feedstuffs to a replacement of animal species by another of lower commercial valu contamination with undesired meat like pig. These results might be usefu effective control of adulterated consumer meat products and violation labeling requirements for meat products. PCR species determination can als used to monitor ruminant feeds for any beef tissue, which has been banne many countries in an effort to control the spread of bovine spongi encephalopathy.

Further studies must be done to applicate animal's species- specific pr on processed meat.

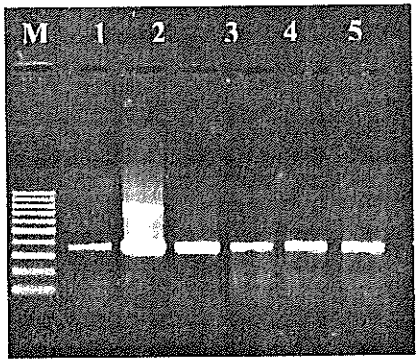

Figure (1): Ethidium bromide stained agarose gel 1.5% electrophoresis showed amplified fragment of 359 bp resulting from amplification of cytochrome b gene generated by common species oligonucleotide primers. Where, lane M: 100 bp DNA marker (100, 200, 300, 400, 500 bp and etc.), lane 1: cattle, lane 2: sheep, lane 3: goat, lane 4: monkey, lane 5: pig and lane 6: dog, respectively.

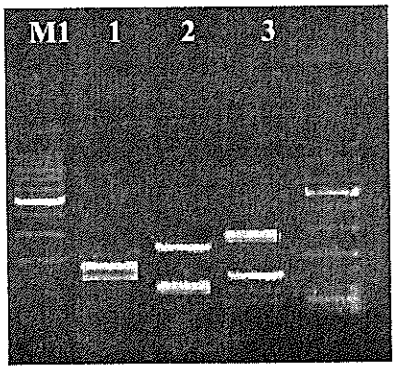

Fig (2) : Ethidium bromide stained agarose gel 2% electrophoresis showed Alu I digest of amplified cytochrome b gene fragments from cattle, pig and monkey muscle.Lane M1 : 100 bp DNA marker (100, 200, 300, 400, 500 bp and etc.). Lanes 1 : cattle, show two bands at 190 and 170 bp. Lane 2 :pig, show two bands at 253, and 74 bp. Lane 3 :monkey, show four bands at 274, 169, 105 and 85 bp. Lane M2 : 50 bp DNA ladder (50, 75, 100, 150, 200, 300, 400 and etc.)

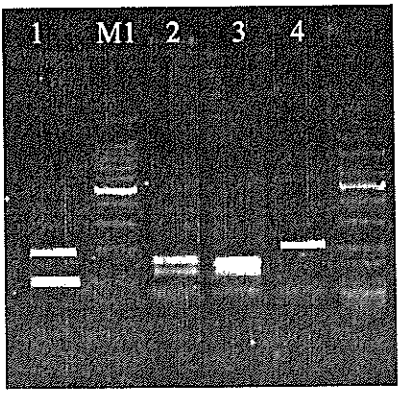

Fig (3) : Ethidium bromide stained agarose gel 2% electrophoresis showed Hinf I digest of amplified cytochrome b gene fragments from cattle muscle and Hae III digest of amplified cytochrome b gene fragments from sheep, pig and goat. Lane 1: cattle, show two bands at 198 and 117 bp. Lanes M1 : 100 bp DNA marker (100, 200, 300, 400, 500 bp and etc.). Lane 2 :sheep, show three bands at 159, 126 and 74 bp. Lane 3 :pig, show two bands at 153 and 74 bp. Lane 4: goat, showed two bands at 239 and 74 bp. Lane M2 : 50 bp DNA ladder (50, 75, 100, 150, 200, 300, 400 and etc.)

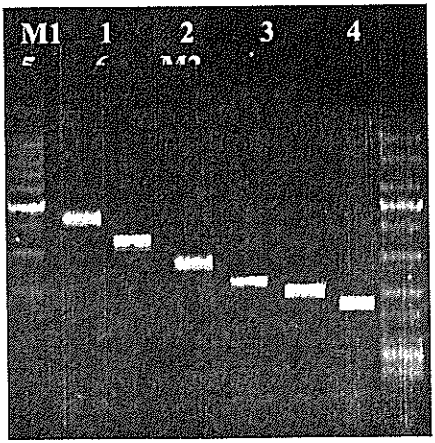

Figure (4): Ethidium bromide stained agarose gel 1.5% electrophoresis showed amplified fragment of mitochondrial DNA with species specific primers Where, lane M1: 100 bp DNA marker (100, 200, 300, 400, 500 bp and etc.). lane 1 - 6: meat samples of donkey, dog, cattle, sheep, pig and goat showed 439, 322, 271,225, 212 and 157 bp respectively. Lane M2: 50 bp ladder (50, 75, 100, 150, 200, 300, 400 and etc.)

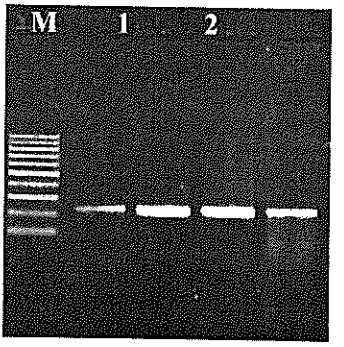

Figure 5: Ethidium bromide stained agarose gel 1.5% electrophoresis showed amplified fragment of 212 bp resulting from amplification of mitochondrial DNA generated by primers specific for pig species. Where, lane M: 100 bp DNA marker (100, 200, 300, 400, 500 bp and etc.), lane 1: pig meat as positive control, lane 2- 5: meat samples of cattle, sheep and goat mixed with meat samples of pig separately respectively.

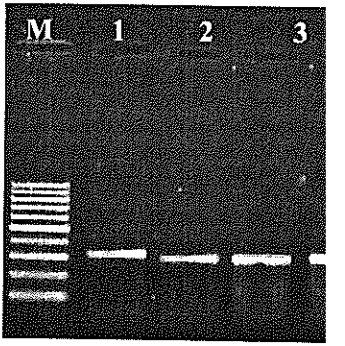

Figure (6): Ethidium bromide st agarose gel 1.5% electrophoresis sh amplified fragment of 322 bp resulting amplification of mitochondrial generated by primers specific for species. Where, lane M: 100 bp DNA m (100, 200, 300, 400, 500 bp and etc.), l dog meat as positive control, lane 2-4: samples of cattle, sheepand goat mixed meat samples of dog separately respec respectively.

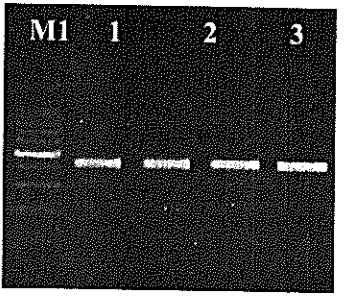

Figure (7): Ethidium bromide stained agarose gel 1.5% electrophoresis showed amplified fragment of 439 bp resulting from amplification of mitochondrial DNA generated by primers specific for horse species. Where, lane M1: 100 bp DNA marker (100, 200, 300, 400, 500 bp and etc.), lane 1:donkey meat as positive control, lane 2- 4: meat samples of cattle, sheep

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ن الاختلاف فى أطوال الأجزاء المقطوعة للسيتوكروم ب جين واختبار إنزيم البلمرة لسل المتخصص لكل من فصائل الحيوانات للتفرقة بين أنواع اللحوم المختلفة

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خلص
رف على نوع اللحوم المستخدمة له دور مهم فى حماية المستهلك من الغش التجارى. لذلك فى هذه الدراسة خدام اختبار إنزيم البلمرة المتسلسل والاختلاف فى الأطوال للأجزاء المقطوعة للسيتوكروم ب جين(-PCR R) للتفرقة بين لحم الأبقار والأغنام والماعز والحمير والكلاب والخنازير وذلك باستخدام بادئ تفاعل خاص كروم ب جين . كانت نتيجة الاختبار إعطاء ٣٥٩ قاعدة نيتروجينية (359 bp) فى كل الأنواع من اللحوم. قطيع هذا الناتج (٣٥٩ قاعدة نيتروجينية) باستخدام إنزيم قاطع Alu I, Hinf I and Hae III كان الناتج عينة من الحيوانات مختلفة عن الأخرى مما يسهل التفرقة بينهم. كما أجرى اختبار إنزيم البلمرة المتسلسل سص باستخدام بادئ تفاعل خاص لكل نوع من الحيوانات وأعطت النتائج ٢٧٥، ٢٢٥، ١٥٧، ٣٤٩، ٣٢٢، قاعدة نيتروجينية لكل من الأبقار والأغنام والماعز والحمير والكلاب والخنازير على التوالى. أظهرت النتائج تخدام كل من الاختبارين نستطيع التفرقة بين اللحوم المختلفة ولكن باستخدام (PCR-RFLP) لن نستطيع ب على أنواع اللحوم عند خلطها مع بعض.