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APPLICATION OF PCR TECHNIQUE FOR ANIMAL SPECIES IDENTIFICATION OF RAW AND HEAT-TREATED MEAT MIXTURES

(With One Table and 4 Figures)

By

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تطبيق اختبار إنزيم البلمرة المتسلسل للتعرف على فصائل الحيوانات فى خليط من اللحوم الخام والمعاملة حراريا

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تم اجراء هذه الدراسة للتأكيد والتعرف على أنواع اللحوم الخام والمعاملة حراريا للحيوانات المختلفة (أبقار، أغنام، ماعز، حمير ، كلاب وخنازير) باستخدام بوادئ خاصة للحوم هذه الحيوانات كل على حدة بالاضافة الى خليط من هذه اللحوم. وكذلك تم استخدام نفس الطريقة على نفس العلوم المعاملة حراريا. تم اسنخدام أطقم من هذه البوادئ الخاصة وأعطت النتائج اجزاء من الحمض الديؤكسى ريبوزى الخاص بالميتوكوندريا عند 271، 225، 157، 349، 322 و 212 قاعدة نيتروجينية لكل من أبقار، أغنام، ماعز، حمير ، كلاب وخنازير كل على حدة. وأوضحت النتائج فى هذه الدراسة امكانية التعرف على لحوم الحيوانات المختلفة سواء الخام أو المصنعة (المعاملة حراريا) باستخدام اختبار تفاعل انزيم البلمرة المتسلسل كطريقة سهلة وسريعة ودقيقة.

SUMMARY

The present study was designed to investigate PCR methods for identification and authentication for different kinds of meat based on species specific PCR. Cattle, sheep, goat, donkey, dog and pig meat were used separately and in meat mixture. The effect of thermal treatment of the sample on PCR technique's ability to identify species was studied through the analysis of microwaved meat mixture. Six sets of species specific primers were used to amplify mitochondrial DNA (mt DNA) specific for cattle, sheep, goat, donkey, dog and pig independently. 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 the above mentioned animal meat respectively. The results obtained in this study demonstrate the suitability of PCR analysis to identify meat of different animal species with the use of species specific primers. Moreover, the technique could be used to identify the species included in animal feed or in meat products submitted to intense heat treatment as it is rapid, reliable, accurate and easy to perform.

Key words: *PCR, meat, meat mixtures, animal species identification, heat-treated meat mixtures.*

INTRODUCTION

Traditionally, animal species identification has been applied mainly for detection of commercial fraud, which involves substitution of an animal species of high commercial value, such as beef, by other species of lower commercial value. It is also a valuable tool for the assessment of risk associated with introduction of animal material that might be harmful to human or animal health such as Bovine spongiform encephalopathy (BSE) (Corona *et al.*, 2007).

The detection of meat species is needed to be performed for other reasons, which includes religious and conservation regulations etc. In India, it becomes especially important due to socio-religious issues associated with the preference. There is religious taboo on the consumption of pork in Muslims and cattle meat in Hindus. There is a ban on the slaughter of cows and bullocks in many states of India. Inspite of ban, cows and bullocks are occasionally slaughtered. Frequently, meat samples are brought to laboratories for identification and confirmation of meat species. Ultimately, the species identification from mixed meat is very critical. However, it is not always possible to differentiate the species by currently available laboratory methods (Rajni, 2007)

The conventionally available methods for species identification from mixed meat include various forms of electrophoresis and use of immune sera in agar gel diffusion. Some of such methods of animal tissue identification are agar gel diffusion, passive haemagglutination, immuno-electrophoresis, counter immunoelectrophoresis, enzyme-linked immunosorbent assay etc. However, the greatest disadvantage of immunological methods for species identification is that the available antisera show cross-reactions. Secondly during cooking the solubility properties and antigens competence of the proteins are altered

considerably. The use of antisera to thermostable antigens has proved to be superior in identification of cooked meat. However, use of such antigens and antisera against them are only partially successful in identification of meats of closely related species of animals like cattle and buffalo from sheep and goats (Bhilegaonkar *et al.,* 1989). In recent past, DNA as a source of information has been used for speciation of meats. DNA based technology for such purpose has several advantages. DNA is more thermostable than many proteins and thus nucleic acids are less liable to be disrupted by processing of foods. It is present in majority of the cells of an organism and therefore, identical information can be obtained from any appropriate sample from the same source, regardless of the tissue of origin. DNA can potentially provide more information than proteins (Corona *et al.,* 2007).

Two major approaches to identify species of meats by DNA techniques are DNA hybridization and PCR based methods. DNA hybridization was the first genetic approach for determination of species identity. In this method, labeled DNA probes were hybridized to samples of genomic DNA covalently attached to nylon membranes in a slot or dot blot form (Baur *et al*., 1989). It was observed that the probes comprising labeled total genomic DNA from a given species would hybridize to DNA from the same species with little cross reactivity. The technique of DNA hybridization has been successfully applied for identification and differentiation of meats of chicken and pork from cooked meats and commercial products (Ebbehoj and Thomsen, 1991). However, meats of closely related species of animals, like sheep and goats showed cross reactivity by this method. In spite of several advantages of DNA hybridization methods for identification and differentiation of meats of different species of animals, these are complicated and generally inadequate.

PCR is a promising approach to species identification. This method is easy, fast and more sensitive. A number of strategies has been employed in PCR including use of repetitive sequences (Calvo *et al.,* 2001), multigene family (Fairbrother *et al.,* 1998) and use of mitochondrial gene (Matsunaga *et al*., 1999) for species identification.

PCR analysis of species-specific mt DNA sequence is the most common method currently being used for species identification (Parodi *et al*., 2002). Detection method based on mt DNA can improve the sensitivity further because each cell has around 104 copies of mt DNA as against just one genomic DNA. Since mt DNA expressed in different species or genera have their evolution specificities, we can identify

individual species by studying mt DNA (Ilhak and Arslan, 2007). Therefore, mt DNA is efficiently used to detect species –specific DNA.

Methods based on DNA amplification are preferred, as they are less affected by industrial processing (Pascoal *et al*., 2005). Generally, mitochondrial DNA (mtDNA) based PCR methods have given good results in analysis of samples submitted to temperature and pressure treatments, in which DNA has been partly degraded (Bottero *et al*., 2003; Rodríguez *et al*., 2004).

The present study was designed to investigate PCR methods for identification and authentication for different kinds of processed cattle, sheep, goat, donkey, dog and pig meat based on species specific PCR to identified processed cattle, sheep, goat, 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 were purchased and collected from Cairo City, Egypt. Meat was wrapped in aluminium foil and cooked in microwave oven at 100 °C for 30 minutes (Shally, 2004). DNA was extracted from each meat sample and stored at –20 ± 1 °C until analyzed.

Test meat mixtures: Each meat samples of cattle, sheep and goat were mixed with meat samples of pig, dog and monkey separately. Following mixing, a 2 gm. portion of each sample was taken separately from each test mixture. DNA was 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). The mixture was incubated at 55°C overnight or until tissue has dissolved. At the second day,100 µl of Protein Precipitation Solution was added to the cell lysate and mix well by vortexing. Then centrifugation 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: 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 Ilhak and Arslan, 2007. The sequence of the primers were illustrated in Table (1).

Table 1: The sequences of six set ofspecies specific oligonucleotide primers used for the detection of cattle, sheep, goat, donkey, dog and pig meats.

	Sequences 5-3	Amplified products
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'	

DNA amplification: DNA amplification was done in 25 ul reaction volume containing 2.5 ul of 10X reaction buffer (65 mM Tris-Hcl, PH 8.8 at 25ºC, 16 mM ammonium sulphate, 200 ug of gelatin per ml), 200 uM of each of four deoxyribonucleotide triphosphates,10 pM of each oligonucleotide primer, 2 mM magnesium chloride, 1 U of Taq DNA

polymerase and 50 ng of template DNA (Johannes *et al.*, 2001). PCR was carried out in a gene cycler (Perken Elmer model 6900).

The optimized cycle program for PCR of denaturation, annealing and extension temperatures was as follow: initial denaturation of 5 min at 94ºC; 30 cycles of 1 minute at 94ºC, 1 minute at 50ºC and 2min. at 72ºC; and final extension step at 72ºC for 5 min (Johannes *et al.*, 2001). After amplification a 5 ul of the reaction product was mixed with 1 ul of 6X gel loading buffer and subjected to electrophoresis on 1.5% agarose gel at 100V for 30 min. Gel were stained with ethiduim bromide and photographed on UV transilluminator (Ilhak and Arslan, 2007). Samples were considered positive when a single band of DNA of PCR amplification product size in cattle's, sheep's, goat's, donkey's, pig's and dog's meat were at 271, 225, 157, 439, 212 and 322 bp, respectively, were evident in the ethidium bromide stained gels compared with the molecular size marker 100-bp DNA ladder (Jena Bioscience Cat. No. M-214). The gels were then photographed using a Polaroid Camera.

RESULTS

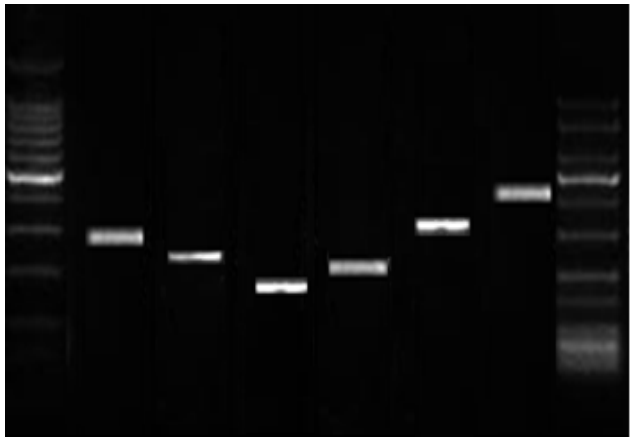

Fig. 1: Ethidium bromide stained agarose gel 1.5% electrophoresis showed amplified fragment of mitochondrial DNA with species specific primers Where, lane M1: 100 bp DNA marker (100, 200, 300, 400, 500 bp and etc.), lane 1 - 6: meat samples of cattle, sheep, goat ,pig, dog and donkey showed 271,225,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

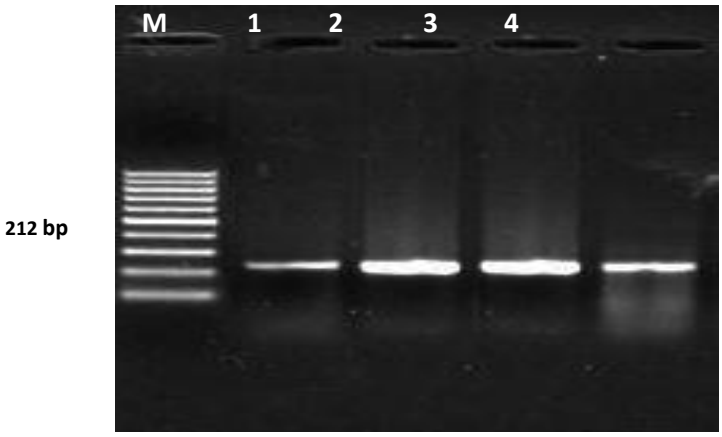

Fig. 2: Ethidium bromide stained agarose gel 1.5% electrophoresis showed amplified fragment of 212 bp resulting from amplification of mitochondrial DNA generated by primers specific for pig species. Where, lane M: 100 bp DNA marker (100, 200, 300, 400, 500 bp and etc.), lane 1: pig meat as positive control,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

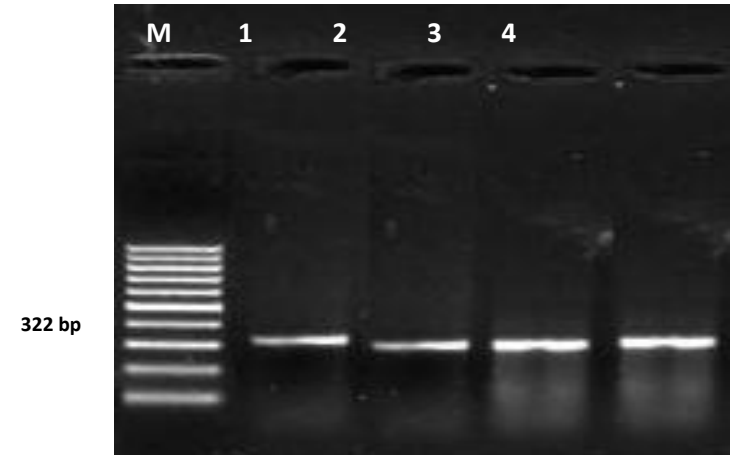

Fig. 3: Ethidium bromide stained agarose gel 1.5% electrophoresis showed amplified fragment of 322 bp resulting from amplification of mitochondrial DNA generated by primers specific for dog species. Where, lane M: 100 bp DNA marker (100, 200, 300, 400, 500 bp and etc.), lane 1: dog meat as positive control, lane 2-4: meat samples of cattle, sheepand goat mixed with meat samples of dog separately respectively respectively.

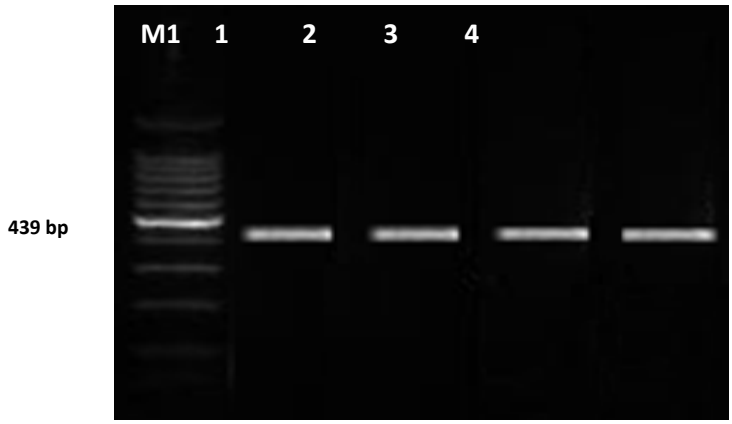

Fig. 4: Ethidium bromide stained agarose gel 1.5% electrophoresis showed amplified fragment of 439 bp resulting from amplification of mitochondrial DNA generated by primers specific for horse species. Where, lane M1: 100 bp DNA marker (100, 200, 300, 400, 500 bp and etc.), lane 1:donkey meat as positive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

DISCUSSION

Nucleic acids present in food are of no nutritional value but are characteristic for the various biological components in complex products. Analysis of specific nucleic acids in food allows the determination of the presence or absence of certain constituents in complex products or the identification of specific characteristics of single food components. As DNA is a rather stable molecule, processed food is generally analyzed using DNA-based method. Because of the high sensitivity, the specificity and rapidity of DNA-based methods, the polymerase chain reaction (PCR) is the method of choice which used in this study.

Six set of species-specific primers were used to amplify mitochondrial DNA (mt DNA) specific for cattle, sheep, goat, horse, dog and pig independently. 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, (Fig. 1). This results confirming that 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. 2), 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. 3). 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. 4). This results 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; 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. 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.

Moreover, Olivier *et al.* (2009) reported that the high forensic value of PCR results is based on the research of specific targets in DNA sequences present in each cell of an organism and conserved at a suitable taxonomic level, commonly at species or groups of animals levels like ruminants or mammals. The detection of multi-copy targets instead of single copy onessuch as mitochondrial DNA is of major interest as it can be present up to hundred of copies per cell depending on the type of tissue.

The effect of thermal treatment of the samples on PCR technique's ability to identify species was studied through the analysis of microwaved meat mixture.

Many a times species identification of cooked meat is warranted. The processing technology (salting, drying, smoking, and cooking) applied during the manufacture of meat products may affect to different extents to the integrity of the extractable DNA. Heat treatments are those steps, which mainly affect the quality of DNA causing its degradation into small size fragments (Dias Neto *et al*., 1994; Martinez and Man., 1998). For this reason, meat samples were cooked in the present study at 100 °C in microwave oven for 30 minutes to simulate cooking. Proper cooking was evident from discolored meat. The high molecular weight DNA could be extracted in sufficient amounts. These results demonstrate the applicability

of DNA techniques for these kinds of samples as previously reported (Koh *et al.*, 1998; Martinez and Man, 1998; Dias Neto *et al.*, 1994; Calvo *et al.*, 2001).

CONCLUSION

The results obtained in this study demonesterate the suitability of PCR analysis to identify meat of different animal species with the use of species – specific primers. Moreover, the technique could be used to identify the species included in animal feed or in meat products submitted to intense heat treatment as it is rapid reliable, accurate and easy to perform. This technique could prove useful for inspection programs intended to assess the species identity of meat products.

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