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Identification of Donkey and Pig Meat in Fresh Minced Beef Mixtures by the Polymerase Chain Reaction

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Abstract: Adulteration of high-priced meat with cheaper meat is one of the most common examples of fraudulence prevalent in meat industry without any consideration of economic, religious or health implications. This work aimed to identify different adulterants (donkey and/or pig) in experimental mixture of fresh minced beef with known formulations by the polymerase chain reaction (PCR). Three meat samples of cattle were mixed respectively with 10% meat of donkey, 10% meat of pig and 10% meat of the both species. These three mixtures and the three individual species were analyzed by PCR for species identification. Agarose gel analysis of PCR product amplified with species-specific primers for the three species showed that mitochondrial DNA (mt DNA) fragments of cattle, donkey and pig meat were respectively 271, 439 and 212 base pair after an amplification of 35 cycles. It is concluded that PCR could be a useful tool for detection of animal species in minced meat when adulterated with more than one different meat species.

Key words: Meat species · Adulteration · Species-specific PCR

INTRODUCTION

Meat species specification needs specialized attention in the system of food quality management. It is a vital field to ensure the food safety to the consumers and it conserves the laws related to meat and meat products. Determining the species origin of different kind of meat samples is important in forensic purpose for species differentiation and identification, it is an integral part of food regulatory issue as adulteration of meat has always been a concern for various reasons such as public health, religious factors, wholesomeness and unhealthy competition in meat market. The adulteration of inferior quality meat into superior quality meat is a common practice all over the world [1, 2, 3]. Minced meat productions remove the morphological characteristics of muscle, making it difficult to identify one type of muscle from another. Because after grinding and mixing, the origin of meat species is easy to conceal in the meat mixture due to the change of meat texture, colour and appearance or even flavour [4, 5]. For this reason, meat substitution with unspecified species, usually of lower quality, is the most common form of economic adulteration in the minced meat industry, constituting a fraudulent act that could have economic and health repercussions. Species substitutions, such as substitution of horsemeat for beef, pork for sheep meat, have been reported in several countries [6, 7]. Minced meat is still facing some unfaithful manufacturing practices and fraud in the form of adulteration with less costly cuts from different animal species. As the physical, chemical and anatomical methods are more suitable for raw meat, minced or comminuted meat requires sophisticated techniques. For some consumer groups, such as Muslims, the contamination of food with meat of pig and its inheritance or any other derived food, dog and cat are forbidden [1]. Another good example of meat adulteration can occur for a variety of reasons often linked to financial gain. Increases in profitability may be achieved by adulteration to improve the perceived quality of products, mimic an established brand and reduce manufacturing costs or for product extension purposes.

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In addition, pork has less nutritional content than others have and increases the health problems [8, 9]. In order to prevent fraud of minced meat in the national and international markets, regulatory authorities and food processing companies are increasingly vigilant and require a rapid and specific analytical procedure for authentication. In the recent past, DNA molecules have been used as target compounds for species identification due to their high stability and unique variability which allow the differentiation of closely related species. DNA identification methods generally give better resolution and confirmatory identification than the traditional morphological or protein identification methods and are the most useful tools for determining animal species in commercial foods and animal products. Among DNA based methods, Polymerase Chain Reaction (PCR) is an effective technique that is highly accurate and relatively fast. The conventional PCR method has a satisfactory performance in the qualitative detection of meat species [10, 11].

Therefore, the main aim of this study was to utilize conventional PCR procedure for detecting different adulterants when they are present at the same time in an experimental mixture of fresh minced beef with known formulations.

MATRIALS AND METHODS

Materials

Meat Samples: Cattle and pig meat samples were purchased from retail markets in Giza and Cairo. Donkey meat was obtained from Surgery Department, Faculty of Veterinary Medicine, Cairo University. Meat samples of different species were transported in an ice box to lab and stored frozen at -20°C until used.

Materials Used for DNA Extraction: DNA extraction using commercial kits (Sigma genomic DNA purification kits).

Materials Used for DNA Amplification: Deoxy nucleotide triphosphates (dNTPs mix as 200 mM/ml) were provided from Promega Company, USA. The species specific primers for amplification of cattle, donkey and pig were obtained from Integrated DNA Technologies, Inc, (Coralville, IA, USA). The sequences and descriptions of oligonucleotide primers were designed from sequence information available in the GenBank database as described by Matsunaga *et al.* [12], Lahiff *et al.* [13] and Ilhak and Arslan [14]. PCR buffer was obtained from Promega Company, USA (500 mM of KCl, 100 mM of Tris –HCl at pH 9.0 and $MgCl_2$ in concentration of 1.5 mM) that was prepared as 10 X concentrations and added to maintain Taq polymerase activity. Taq DNA polymerase was obtained from Promega, Madison, WI, USA (50% glycerol and 50Mm of EDTA at concentration 5 units/µl in buffer) that polymerized nucleotides to DNA at 72°C and remained function at 95°C. Light mineral oil was obtained from Amersco, Cleavland, Ohio, USA and used to cover the reaction mixture to prevent evaporation.

Materials Used for Gel Electrophoresis: Agarose gel was obtained from International New Technologies in New Hoven, Connecticut and USA. It was free from DNase and RNase and prepared as 1.5% concentration. Tris- EDTA buffer (TE buffer) was obtained from Amersco, Cleavland, Ohio, USA (Tris –HCl and 1.0mM of EDTA at pH 8.0) that used for preparation of electrophoresis buffer. Loading buffer obtained from Amersco, Cleavland, Ohio, USA (xylene, cyanide, glycerol and 1% Bromophenol as 6x concentration). It was used for deposition of PCR products in the gel wells. Ethidium bromide (2.7 Diamino-10ethyle-9-phenyl phenathridium bromide) obtained from Sigma, Aldrish, Germany. 10 mg/ml of ethidium bromide was dissolved in bi distilled water for using as a fluorescent dye to stain gel electrphorsed DNA during examination by ultraviolet transillumination. Molecular marker (DNA ladder) weight marker 50bp was supplied by GenDirex, USA. The expected amplicon size (bp) of cattle, donkey and pig were 271, 439 and 212 base pair (bp), respectively.

Methods

Samples Preparation: Cattle, donkey and pig samples were thawed, cut into small cubes and minced by an electric homogenizer to ensure a homogeneous mixture. Homogenizer was carefully washed between each preparation by distilled water and dried with a tissue paper. Three individual species (100g cattle, 100g donkey and 100g pig meats) were prepared then three mixtures of them were prepared. The first mixture sample composed of 90g cattle meat mixed with 10g donkey. The second mixture sample composed of 90g cattle meat mixed with 10g pig meat while the third mixture sample composed of 80g cattle meat mixed with 10g donkey meat and 10g pig meat.

DNA Extraction from Meat Mixture Samples: Portion from each sample (2g) was taken separately for DNA extraction as described by Koh *et al.* [15], with a slight

modification [14]. Each meat sample was homogenized in 15ml polypropylene with 4 ml of TNES solution as digestion buffer, incubated with shaking at 50°C for 12-18 hours in tightly capped tubes and cooled at room temperature then 0.1µl of RNase was added at 37°C for an hour. In a 1.5 ml Eppendorf tube, A 750-µl aliquot of the resulting homogenate was added to 10µl of proteinase K and 50µl of 10% SDS to lysate and mixed by inverting several times. The mixture was shaken vigorously and kept for 8 h at 58°C in a water bath. A 250-µl of 6 M NaCl was added to the mixture and then was centrifuged at 11600 xg for 5 min. A 500-µl portion of the aquatic phase of the sample was transferred into a separate Eppendorf tube and 300µl of a phenol-chloroform-isoamyl alcohol (25:24:1) mixture was added, followed by vigorous shaking and centrifugation at 11600 xg for 5 min. A 400-µl portion of the upper layer was then transferred into another tube and 300µl of chloroform was added, followed by mixing and centrifugation. A 300-µl portion of the upper phase was then taken and 400µl of absolute ethanol at –20°C and 40µl of sodium acetate were added prior to vortexing and storing the sample at –20°C for 8 h for precipitation of DNA. The resulting mixture was then centrifuged at 11600 xg for 10 min and then the liquid phase was removed. A 400-ml volume of 70% ethanol was added to the pellet, followed by centrifugation at 11600 xg for 5 min for washing of the DNA. Finally, ethanol was removed and the tube containing DNA was held at room temperature for 30 min for further removal of the residual ethanol via evaporation. The pellet, which was the extracted DNA, was diluted with 100µl of sterile dH2O and used for PCR reaction.

Polymerase Chain Reaction (PCR): The 50-µl reaction mixture was prepared in an Eppendorf tube containing 5µl of 10 X PCR buffer, 5µl of 25 mM $MgCl_2$, 250 µM (dNTP), 0.25µl of Taq DNA polymerase, 20 pmol of each forward and reverse primer, 5µl of target DNA and remaining nuclease-free water. PCR was optimized with different annealing temperatures. The optimal annealing temperature was 58 °C for all primers. Each cycle holding at 94°C for 45 s, at 58°C for 45 s and at 72°C for 90 seconds for 35 cycles PCR amplification.

Electrophoresis: Electrophoresis was run on agarose gel (1.5%) at 100 V for 2 h on a 15µl portion of the amplified DNA fragments. The resulting gel was stained with ethidium bromide (0.5µg/ml), visualized using a UV transilluminator and photographed with a digital camera. The experiments were conducted in triplicate.

RESULTS AND DISCUSSION

The adulteration by substitution of meat has always been a concern for various reasons such as public health, religious factors, wholesomeness and unhealthy competition in meat market. Of these methods, DNA-based methods offer the greatest potential because they are stable and not tissue dependent. In the present study, agarose gel analysis of PCR product amplified with species specific primers in individual cattle meat sample showed that mitochondrial DNA fragments of cattle meat was 271 bp after an amplification of 35 cycles as shown in Fig. 1. This approach was earlier employed by many other workers for further confirmation of PCR products amplified from meat and meat products [16]. All the DNA samples were found free of proteins and RNA. Universal primers have been designed to amplify the conserved region of cytochrome-b gene in more than 100 species, including mammals, birds, amphibians, fishes and some invertebrates [12]. Mitochondrial cytochrome-b gene based PCR was chosen because the mitochondrial genome is easy to isolate from the nuclear genome, present in high number of copies, smaller size and rapid accumulation of mutations and the post PCR analysis is much simpler that agreed with Wilson *et al.* [17]. PCR detection depends on the detection of the specific DNA molecules which is a relatively stable molecule allowing analysis of processed and heat treated food products [18].

The obtained results illustrated that individual donkey meat sample could be detected by agarose gel analysis of PCR product amplified with species-specific primers. Mitochondrial DNA (mt DNA), a fragment of 439 bp, of donkey meat was detected (Fig. 2). Numerous reports showed that species-specific PCR assay is rapid and cost effective for identification of meat species due to specific detection of target sequence without the need of further sequencing or digestion of the PCR products with restriction enzymes. It is successfully used for identification of various species of meat [19, 20]. Also, Chisholm *et al.* [21] developed real-time PCR assays specific for horse and donkey, applicable for detection of low levels of horse or donkey meat in commercial products. Primers, designed to the mitochondrial cytochrome-b gene, were 30 mismatched to closely related and other commercial species. Both assays were highly sensitive and detected the presence of 1 pg of donkey template DNA or 25 pg of horse template DNA when assessed using dilutions of DNA in water. DNA extraction of individual pig meat sample was performed with high quality and quantity and amplification with

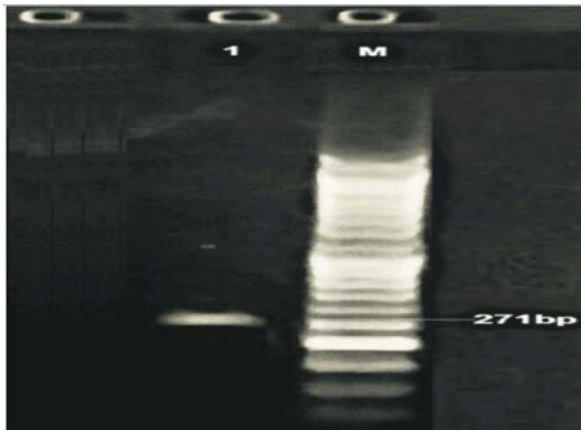

Fig. 1: Agarose gel analysis of PCR product amplified with species-specific primers. M: molecular marker (50 bp), 1: cattle meat (271bp)

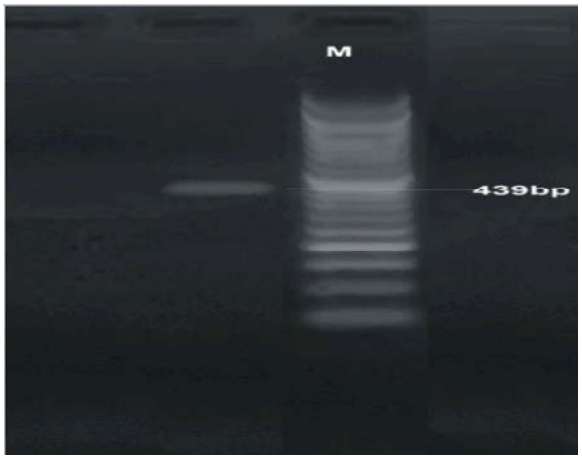

Fig. 2: Agarose gel analysis of PCR product amplified with species-specific primers. M: molecular marker (50 bp), 1: donkey meat (439bp)

Fig. 3: Agarose gel analysis of PCR product amplified with species-specific primers. M: molecular marker (50 bp), 1: pig meat (212bp)

designed specific primers showed an amplicon with a length of 212 bp (Fig. 3). The used of PCR techniques to amplify the mtDNA of cyt b in identifying pig DNA in foods has been reported by Kesmen *et al.* [22] and Chandrika *et al.* [23]. A detection based on mtDNA is popular due to its different specificity expressed in the species or genera through the study of mtDNA. There are approximately 104 copies of mtDNA available per cell compared to only one copy of genomic DNA. Thus it is more efficient to detect species-specific DNA using mtDNA than genomic DNA [24, 25]. In addition, Aida *et al.* [26] developed a method for species identification from pork and lard samples using PCR analysis of a conserved region in the mitochondrial cytochrome-b gene. The amplified PCR products were cut with restriction enzyme resulting in porcine specific restriction fragment length polymorphisms that yielded excellent results for identification of pig species. It is a potentially reliable technique for detection of pig meat and fat from other animals for Halal authentication. On the other side, Kesmen *et al.* [27], Dooley *et al.* [28] and Martín *et al.* [29] recorded that appearance of a pig-specific product of 712 bp is conclusive for pork in line with other pig-specific real time PCR assays that are based on either TaqMan or SyBr-Green.

In the present study, PCR products were examined for its specificity to meat species by identification of the corresponding species. The PCR products in first mixture meat sample showed species specific DNA fragments of 271 and 439 bp which related to cattle and donkey meats, respectively as shown in Fig. 4. Results of the present study was supported with the findings of Meyer *et al.* [30], Hopwood *et al.* [31] and Partis *et al.* [32], who reported that PCR could be used for identification of meat mixes at 1% and 0.5% levels. On the other side, PCR products of the second mixture meat sample showed species specific DNA fragments of 271 and 212 bp related to cattle and pig, respectively as illustrated in Fig. 5. Results of the present study supported the findings of many publishers who detected 0.5% pork in beef using the duplex PCR technique. Their results revealed that PCR was the method of choice for identifying meat species in muscle foods. In addition, detection of pork by PCR has been reported previously by targeting nuclear as well as mitochondrial DNA sequences [30, 33]. Furthermore, Yoshida *et al.* [34] applied PCR using specific primers and mitochondrial oligonucleotide primers for detection of pork and their derivatives in processed food products even damage or fragmented DNA. Recently, Hamzah *et al.* [35] could detect the presence of porcine DNA in meat

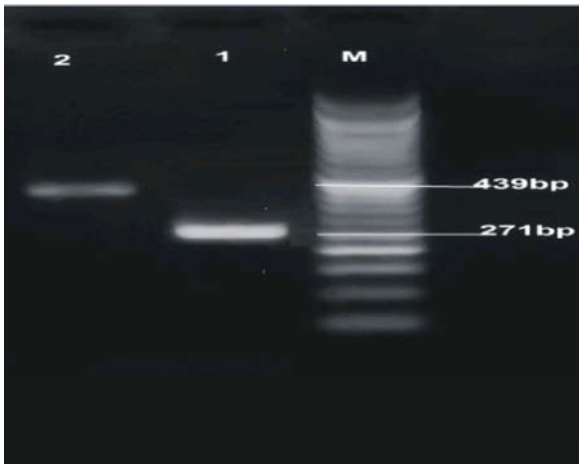

Fig. 4: Agarose gel analysis of PCR product amplified with species-specific primers. M: molecular marker (50 bp), 1: cattle meat (271bp), 2: donkey meat (439bp)

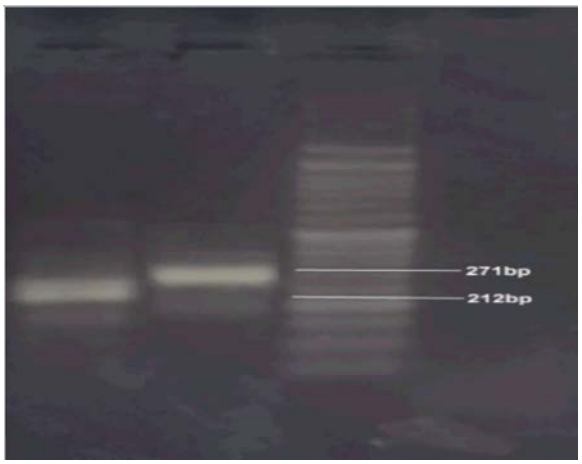

Fig. 5: Agarose gel analysis of PCR product amplified with species-specific primers. M: molecular marker (50 bp), 1: pig meat (212bp), 2: cattle meat (271bp)

products in the market using different mitochondrial (*mt*) DNA on conventional PCR. DNA of raw pork meat was used as a positive control, while nucleus free water is used as negative control. DNA of meat products was amplified by using species-specific primer namely *mtATP6* with band size of 83-bp and Pork1 and Pork2 with band size of 531-base pair (bp) mitochondrial (*mt*) DNA D-loop primer to detect pork species.

The identification of animal species in meat and meat products has proved to be difficult, particularly in samples of complex composition. Using of target DNA was successfully identified for each species tested. Agarose gel analysis of PCR product amplified with species specific primers of third mixture meat sample showed

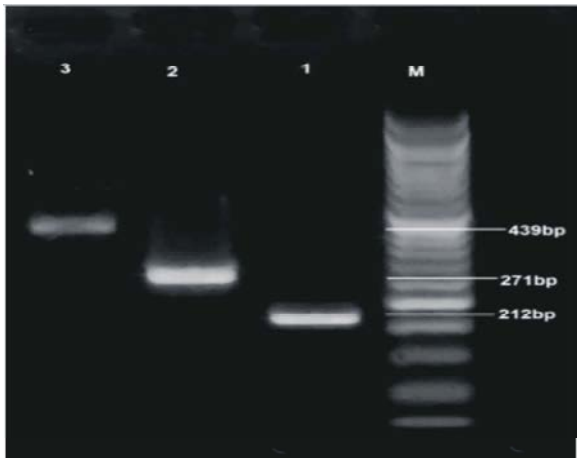

Fig. 6: Agarose gel analysis of PCR product amplified with species-specific primers. M: molecular marker (50 bp), 1: pig meat (212bp), 2: cattle meat (271bp), 3: donkey meat (439bp)

mitochondrial DNA fragments of 271, 439 and 212bp which belong to cattle, donkey and pig (Fig. 6). Meyer *et al.* [30] reported that PCR products were examined for its specificity to meat species by identification of the corresponding species. The products showed species specific DNA fragments of 420, 343 and 350 bp from chicken, pork and donkey meats, respectively. Beef, chicken, pork and donkey can be qualitatively identified and differentiated by PCR. This method can be applied with equal efficiency to both fresh and processed meats. Kumar *et al.* [36] used Cytochrome b gene based in PCR to identify and differentiate the cooked meat of sheep, goat, cattle, pig and chicken. The PCR products from cooked meat samples of these animal species produced DNA fragments of 331, 157, 274, 398 and 227 base pairs respectively and clearly differentiated the species origin of meat. On the contrary, Doosti *et al.* [37] mentioned that Amplification with species-specific oligonucleotide primers revealed a 271, 274, 149, 266 and 221 bp from bovine, sheep, pork, chicken and both donkey and horse genomic DNA, respectively The amplification of mitochondrial DNA segment (cytochrome-b gene) in both donkey and horse yielded the same amplicon with a size of 359 bp. The PCR amplification size and the position of the PCR with species specific oligonucleotide primers and the mitochondrial DNA segment (221 bp and 359 bp) with both donkey and horse are exactly same. Haining *et al.* [38] developed quadruple multiplex PCR assay for meat (beef, duck, mutton and pork) in processed meat products. By mixing four primers in appropriate ratios could be identified by

the PCR. A forward primer was designed on a conserved DNA sequence in the mitochondrial ND2 and 16S rDNA gene in sheep and duck genes and reverse primers on species-specific DNA sequences for each species. PCR primers were designed to give different length fragments from the four meats. The products showed species-specific DNA fragments of 116, 212, 177 and 322 bp from beef, pork, mutton and duck. Optimal PCR conditions were established. The assay sensitivity under these conditions was 0.1ng and its specificity was 100 %. The results of the study suggest that PCR represents a simple, efficient test method as a practical alternative for the rapid detection and identification of meat.

CONCLUSION

It can be concluded that adulteration is a serious food safety and quality issue with an increasing prevalence in meat and meat products all over the world. PCR based method is highly sensitive and specific for detection of meat species and also can detect very small amount of adulteration. It is expected that this technique a useful laboratory tool for species identification, especially for meat traceability. The species-specific PCR amplification yielded excellent results for identification of pork derivatives in food products and it is a potentially reliable and suitable technique in routine food analysis for Halal certification.

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