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Use of molecular biology techniques in the detection of fraud meat in the Egyptian market

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Food safety and quality are major concerns and any case of food adulteration has a great impact on public opinion. Identification of animal species used in commercial meat products is important with respect to economic and sanitary issues. The aim of this research was to detect ruminant and equine species in minced meat and Egyptian sausage using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Species differentiation was performed by digestion of PCR products with Tsp509I and AluI restriction enzymes. Our results indicate that 4 (4%), 3 (3%) and 5 (5%) of examined samples (100) were contaminated with sheep, goat and donkey meat, respectively. These results indicate that 12% of examined samples were adulterated, although they were labeled as beef meat 100%. It can be concluded that molecular methods such as PCR and PCR-RFLP are potentially powerful and reliable techniques for detection of adulteration with different meat species in meat products.

Key words: Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), *Cytochrome-b* gene, identification, adulteration, meat products.

INTRODUCTION

Meat species specification is an area which needs specialized attention in the food quality management system. It is a vital field to ensure food safety (Singh and Neelam, 2011). In developing countries, there is an increasing demand for meat products (Delgado, 2003). There are different aspects that interfere in the selection of meat products including price, quality and nutritional attributes.

Nowadays consumers are increasingly aware of their health and are looking for more comprehensive information on the safety of the foods they consume (Verbeke and Ward, 2006). In spite of the food labeling

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regulations, the adulteration or misrepresentation of food products for more financial gain is a common practice all over the world (Shears, 2010; Doosti et al., 2014). Thus authentic testing of meat products avoids unfair market competition and protects consumers from fraudulent practices of meat adulteration.

Although historically, meat has not been widely associated with adulteration because of the recognizable joints (Nakyinsige et al., 2012), but in processed meat products, meat species substitution occurs more regularly (Ayaz et al., 2006). The substitution with cheaper species is very difficult to detect in such products by visual observation after grinding and/or heat processing (Abd El-Nasser et al., 2010). Also accidental cross contamination of meat products may occur during processing, due to improper handling and using shared equipments (Ilhak and Arslan, 2007).

Developments in molecular biology have facilitated identification of plant, bacteria, and animal species with high accuracy. Recently, the protein-based and DNA-based techniques were widely used to identify prohibited species in food products.

Protein-based technique includes the immunological (Lopez-Calleja et al., 2007), electrophoretical (Mayer, 2005), chromatography such as high performance liquid chromatography (HPLC) and DNA-based methods, including DNA hybridization (Hunt, 1997), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis (Mane et al., 2014), species-specific PCR (Haunshi et al., 2009), multiplex PCR (Ghovvati et al., 2009) and real-time PCR (Walker et al., 2013). The advantage of PCR based tests are higher accuracy, time-saving, high sensitivity and flexibility, compared to other methods.

PCR-RFLP is a two-step reaction to identify multiple species after restriction enzyme digestion of PCR amplified DNA sequence (Haider et al., 2012). Therefore, in this study, PCR and PCR-RFLP techniques were done for the detection of adulteration and identification of sheep, goat, donkey and horse's meat using species-specific oligonucleotide primers.

MATERIALS AND METHODS

Sample collection and preparation

One hundred (100) samples of beef meat (50 of minced meat and 50 of sausages) from popular retail markets in Cairo and Giza governorates were analyzed for detection of meat adulteration. The samples were stored at -20°C until used for DNA extraction in order to prevent the enzymatic degradation of DNA. The samples were prepared based on the method of Santaclara et al. (2007). Sausage samples were extracted by suspending in methanol-chloroform-water (2:1:0.8) solution for 2 h to prevent the oil disturbance in DNA extraction process.

Afterward, the supernatant was discarded and the samples were washed once using ultrapure (1 ml) water to eliminate the remnants of the used solution.

DNA extraction

The extraction of mitochondrial DNA from all samples was performed using tissue mini kit (QIAGEN, Hilden, Germany). The procedure followed the manufacturer's instruction. Concentration and purity of DNA were also assessed by NanoDrop™ ND-2000 spectrophotometry (Thermo, Wilmington, USA).

Species-specific primers and PCR amplification

The mitochondrial DNA segment (cytochrome-b gene) in cattle, sheep, goat, donkey and horse were amplified with the use of primer sequences as described in Table 1. Universal primers CB7u used in this study were targeted 195 bp fragment of variable region of mitochondrial cytochrome-b, while primers of the Donkey's and Horse's cytochrome-b were targeted 359 bp fragment of variable region of mitochondrial cytochrome-b (Doosti et al., 2014).

PCR procedure

Amplification of species fragments was carried out in a total volume of 25 µL containing 12.5 µL of taq master mix (2x) (Invitrogen, California, USA), 0.1 mg/ml bovine serum albumin (BSA) (Roche, Mannhein, Germany), 10 pmol of each primer and 50 ng of template DNA. The volume was completed using DNase and RNase /free distilled water.

Amplification was performed in an Piometra personal thermal cycler with the following cycling conditions; after an initial heat denaturation step at 94°C for 2 min, 35 cycles were programmed as follows: 94°C for 1 min, 60°C (Universal primers CB7u) or 58°C (primers Donkey's and Horse's cytochrome-b) for 1 min, 72°C for 1 min and final extension step at 72°C for 5 min. The PCR products were analyzed by electrophoresis on 2% agarose gel (Invitrogen, California, USA) run in TBE 1X Buffer for 80 min at 90 V and stained with ethidium bromide (10 µg/L) for 20 min.

RFLP analysis

Five microliters of PCR product, 2 units of restriction enzyme Tsp509I or AluI restriction enzyme (Faenzyme,) (Fermentas, USA), 0.1 µL BSA (1 mg/ml) and 2 µL of 10X reaction buffer at the final volume of 20 µL were incubated for 2.5 h at 55°C and it was inactivated at 65°C for 20 min. Digestion products were separated by electrophoresis on non-denaturing 8% polyacrylamide gel stained with AgNO3 and photographed by Fugi digital camera (Fine pix S5700, Japan).

Irradiation process

Adulterated meat samples were subjected to three different doses of gamma irradiation (1.5, 3 and 5 kGy) at dose rate 3.49 kGy/ h using the "Indian Gamma Chamber 4000 A" with a ^{60}Co source. The irradiation process was conducted at the National Center for Radiation Research and Technology (NCRRT), Nasr city, Cairo, Egypt. After irradiation, adulterated samples were kept at -20°C until used for DNA extraction repeating all previous steps.

RESULTS AND DISCUSSION

Identifying meat species used in meat products is a critical point in the quality control measures. Molecular

Table 1. Primer sequences of species-specific DNA regions and their annealing temperatures.

DNA regions	Primer sequence 5 to 3	Annealing temperature (°C)
CB7u *cytochrome-b*	GCGTACGCAATCTTACGATCA CTGGCCTCCAATTCATGTGAG	60
Donkey's and Horse's *cytochrome-b*	CCATCCAACATCTCAGCATGATGAAA GCCCCTCAGAATGATATTTGTCCTCA	58

Table 2. Fragments lengths for ruminant species, donkey and horse after digestion of the PCR products with restriction enzyme.

Species	Amplicon (bp)	Restriction enzymes	Fragment length (bp)
Sheep	193	Tsp509I	13, 75, 105
Goat	195		13, 182
Cattle	185		3, 68, 114
Donkey and horse	359	AluI	-
Horse	359		74, 96, 189

Table 3. Number and % of adulterated minced meat and sausage samples (n= 50 each).

Sample	Number of adulterated samples with			Percentage		
	Sheep	Goat	Donkey	Sheep	Goat	Donkey
Minced meat (n=50)	2	1	2	4	2	4
Sausage (n=50)	2	2	3	4	4	6

techniques have been used elsewhere for meat species identification. The PCR assays targets genomic, as well as, mitochondrial DNA for the purpose of meat species identification. The conventional techniques allow the qualitative detection of different species with a defined limit of detection. However, Real-time PCR generally offers greater sensitivity and specificity and is a quantitative method for identification of species (Walker et al., 2013). Species-specific PCR assay was found to be 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 (Rodriguez et al., 2004), but it cannot be designed when species are very closely related (Kelly et al., 2003). On the contrary, PCR-RFLP could differentiate closely related meat species (Amjadi et al., 2012; Jaayid, 2013; Mane et al., 2014).

The amplification of species-specific DNA segments and restriction fragment length polymorphisms in cattle, sheep, goat, horse and donkey are represented in Tables 2 and 3 as well as Figures 1and 2. The CB7u primer produced specific fragments of about 195 bp for ruminant species. The size of ruminant fragments depends on the number of deletions in each species sequence (Burger et al., 2002). In this study, Cyt-b gene sequence from mitochondrial DNA was used for ruminant and equine DNAs identification and detection in meat because, mitochondrial DNA has numerous copies per each cell and it can provide the sequence variety for identification of closely related species faster than genomic DNA (Bellis et al., 2003). The suitability of cyt *b* gene sequence analysis to verify the claimed origin of supplied meat on a routine basis was previously confirmed (Jaayid, 2013, Doosti et al., 2014). The PCR-RFLP technique was able to distinguish between meat of animals that belong to the same family or same species such as the bovine family or the equine family (Doosti et al., 2014). Discrimination between donkey and horse's meat was previously reported by several investigators (Abdel-Rahman et al., 2009; Jaayid, 2013), using *Alu*I restriction enzyme, three fragments (189-, 96- and 74-bp) from the amplified gene encoding *cytochrome-b* gene (359 bp) were obtained in horse, whereas in donkey meat samples no fragments were obtained.

Results of RFLP assay (Table 3) reveal that 2/50 (4%) (Figure 2 lane 2), 1/50 (2%) (Figure 1 lane 6), and 2/50 (4%) of minced meat samples were contaminated with sheep, goat and donkey, contrary to what was mentioned

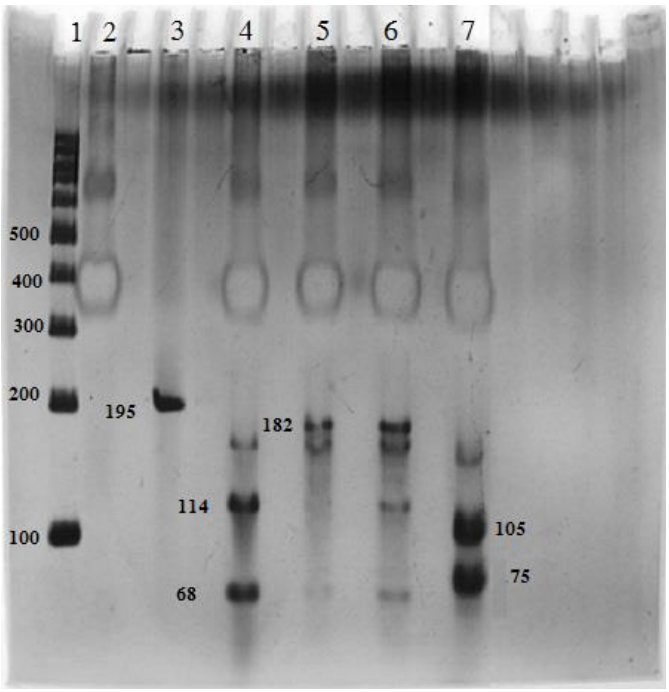

Figure 1. Polyacrylamide gel revealing restriction profiles of 195 bp Cyt-b PCR products obtained after treatment with Tsp509I. Lane 1 marker; lane 2, negative control; lane 3, positive control (undigested); lane 4, control cattle meat; lane 5, control goat meat; lane 6, commercial minced meat; Lane 7, control sheep meat.

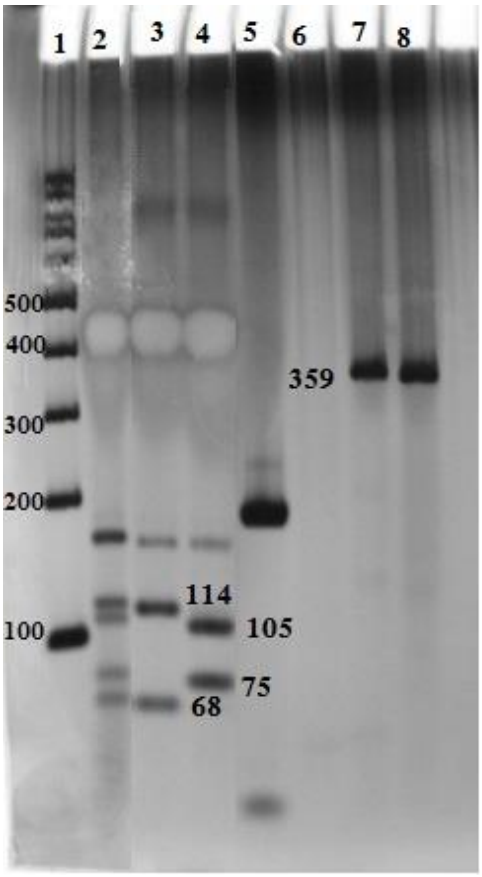

Figure 2. Polyacrylamide gel revealing restriction profiles of 195 bp Cyt-b PCR products obtained after treatment with Tsp509I for lanes 2-4. Lane 1, marker; lane 2, commercial minced meat; lane 3, control cattle meat; lane 4, control sheep meat; lane 5, positive control (undigested); lane 6, negative control; lane 7, control donkey meat; lane 8, PCR of primer of Donkey's and Horse's *cytochrome-b* after treatment with AluI restriction enzyme.

on their labels. Similarly 2/50 (4%), 2/50 (4%), 3/50 (6%) (Figure 2 lane 7, 8) of sausage samples were contaminated with sheep, goat and donkey. In total, 12% of the collected samples were contaminated with sheep, goat and donkey in contrast to what was labeled. Adulteration with donkey and horse meat was previously reported by several investigators (Jaayid, 2013; El-Shewy, 2007; Abd El-Nasser et al., 2010). The adulteration rate with donkey meat in our study was less than that reported in Assuit governorate in Egypt by Abd El-Nasser et al. (2010) in minced meat (7%) and sausage (8%); this may be due to the stringent control on food in Cairo, the Capital of Egypt. Donkey is not a species commercially used for human consumption. Its presence indicates adulteration for economic gain and so gives an idea that meat has been processed under non-sanitary conditions representing potential risks to human health. Adulteration of minced meat with sheep (60%) and goat (10%) was reported by Amjadi et al. (2012) with rate higher than that reported in our study.

Fraud in meat based products by adding low quality and cheap meat has always been an issue in food industries. The PCR-RFLP method used in this research, is cost-effective, reliable and very specific to be applied on food products that have endured different handling conditions during preparation process. It should play an important role in the food industry regulation and legal issues.

By irradiating adulterated meat with 1.5, 3 and 5 kGy, there was no change in the results of PCR and PCR-RELP. This finding was justified by the Council for Agricultural Science and Technology (CAST, 1989) by estimating that a dose of 1 kGy would break fewer than 10 chemical bonds for every ten million bonds present, an extremely small percentage. Cooking, or applying infrared radiation to foods, produces similar changes in chemical bonds.

It can be concluded from the findings of the present study that molecular methods such as PCR and PCR-RFLP are potentially reliable techniques for the detection of different meat species in meat products.

Conflict of interests

The authors have not declared any conflict of interests.

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