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Full Length Research Paper

Development of rapid PCR-RFLP technique for identification of sheep, cattle and goat's species and fraud detection in Iranian commercial meat products

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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 realize ruminant species in meat products using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The universal CB7u primers pair was used for amplifying ~195 bp fragment from a variable region of cytochrome-*b* mitochondrial DNA gene by polymerase chain reaction. Species differentiation was realized by digestion of the amplified ~195 bp fragments with *Sse*9I restriction enzyme. The results indicate that 7/7 of Kebab loghmeh, 9/10 of minced meat, 4/8 of beef burger and 2/5 samples of canned stew samples, were contaminated with one of prohibited ruminant species residual. Furthermore, the results reveal that 5/30 of samples had cross-contamination with a mixture of meat originated from various species, which was against the labelled nutrition information. Our results indicate that the PCR-RFLP technique is a powerful and reproducible test for detection and separation of ruminant species residuals in commercial meat products, especially in developing countries.

Key words: Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), mitochondrial DNA, ruminant species, commercial meat products, cytochrome-*b* gene.

INTRODUCTION

Species identification in food products by amplification of mitochondrial DNA was increasingly employed during the last decades. Identification of animal species used in meat products from consumer point of view is very important with respect to economic, religious and sanitary issues (Meyer et al., 1995). Sometimes, labels of the food products may not show their real contents and sometimes ingredients separation of food products through physical or chemical tests may not always be possible. Therefore, special methods are required to determine and authenticate food products to support consumers against any possible fraud. The European Union controls the foods' safety by establishing stringent laws for food products labeling (Di Pinto et al., 2005).

Recently, the protein-based and DNA-based techniques were widely used to identify prohibited species in food products. Protein-based technique includes the immunological (Haza et al., 1999; Lopez-Calleja et al., 2007), electrophoretical (Addeo et al., 1995; Mayer, 2005), chromatography such as high performance liquid chromatography (HPLC) and DNA-based methods, including DNA hybridization (Armstrong et al., 1992; Ebbehoj and Thomsen, 1991; Hunt et al., 1997;

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Table 1. Samples submitted for the assay.

Species	Positive control
Bos taurus	+
Capra hircus	+
Ovis aries	+
Equus caballus	-
Equus asinus	-
Gallus gallus	-

Knuutinen and Harjula, 1998; Mayer et al., 1997), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis (Fajardo et al., 2009; Murugaiah et al., 2009), species-specific PCR (Che Man et al., 2007; Haunshi et al., 2009), multiplex PCR (Bottero et al., 2003; Ghovvati et al., 2009) and real-time PCR (Dalmasso et al., 2007; Zhang et al., 2007). The advantage of PCR based tests are higher accuracy, time-saving, high sensitivity and flexibility compared to other methods.

The main objective of this study was to introduce a new PCR-based method to identify and detect animal species in food products. Hence, PCR-RFLP method was optimized and successfully used along with one restriction enzyme to identify contamination of ruminant species (cow, sheep and goat) and fraud detection in some Iranian commercial meat products.

MATERIALS AND METHODS

Sample collection and preparation

Samples of raw meat and autoclaved treated meat (121°C for 15 min) from three ruminant species and three non-ruminant species (equine and poultry) were analyzed as positive and negative controls, respectively. The study species are presented in Table 1. Four types of commercial meat products including minced meat (N = 8), Kebab loghmeh (N = 10), beef burgers (N = 7) and canned stew (N = 5) obtained from different companies were collected in Mashhad and Tehran, in Iran. All of these products were labeled (beef ingredient) and 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 Santaclara et al. (2007) method.

DNA extraction from autoclaved samples did not require any preparation. The oil in minced meat, Kebab loghmeh, beef burgers and canned stew samples was 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 by ultrapure 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 was followed according to the manufacturer's instruction. Concentration and purity of DNA were also assessed by NanoDrop™ ND-2000 spectrophotometry (Thermo, Wilmington, USA).

Oligonucleotide primers and PCR amplification

Two universal primers CB7u (5'-GCG TAC GCA ATC TTA CGA TCA A-3') and CB7l (5'-CTG GCC TCC AAT TCA TGT GAG-3') used in this study were targeted ~195 bp fragment of variable region of mitochondrial cytochrome-*b* (Burger et al., 2002). Amplification of species fragments was carried out in a total volume of 25 µL containing 75 mM Tris–HCl (pH 8.8), 1 U of platinum *Taq* DNA polymerase (Invitrogen, California, USA), 0.1 mg/ml bovine serum albumin (BSA) (Roche, Mannhein, Germany), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Pharmacia, Uppsala, Sweden), 2 mM $MgCl_2$, 10 pmol of each primer and 50 ng of template DNA. Amplification was performed in an ABI 9700 thermal cycler (Applied Biosystems, California, USA) 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 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 ng/ml) for 20 min. The size of the amplified fragments was estimated by M50 and M100 molecular marker (Iso Gene, Moscow, Russia).

RFLP analysis

5 µl of PCR product, 2 U of restriction enzyme *Sse*9I (Sibenzyme, Moscow, Russia), 0.1 µL BSA (1 mg/ml) and 2 µL of 10X reaction buffer (Sibenzyme, Moscow, Russia) 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 $AgNO_3$. The obtained fragment lengths are shown in Table 2.

RESULTS AND DISCUSSION

DNA extraction

Many studies have evaluated the efficiency of various methods of DNA extraction from food materials (Ghovvati et al., 2009). In this research, DNA extraction was based on the binding of DNA to silica matrix in the presence of chaotropic agents. This technique was considered effective for elimination of PCR inhibitors that might interfere with polymerase chain reaction. The result demonstrates that extracted DNA was sufficient for Cyt-b

Table 2. Fragments lengths for ruminant species in this study after digestion of the PCR products with restriction enzyme *Sse*9I.

Species	Amplicon (bp)	Fragment length (bp)
Ovis aries (sheep)	193	13, 75, 105
Capra hircus (goat)	195	13,182
Bos taurus (cattle)	185	3, 68, 114

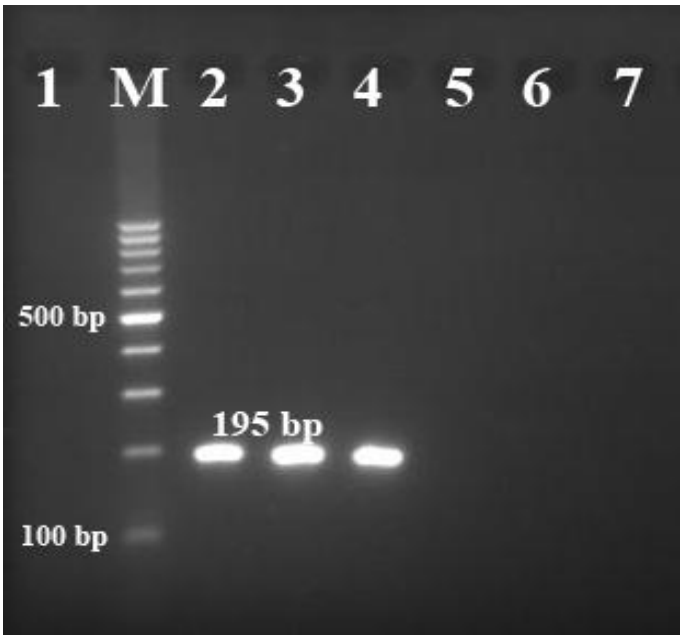

Figure 1. Specificity of primers for amplification of ~195 bp fragments of various species under varying conditions, Lane 1, Negative control reagent; lane 2, *Capra hircus* DNA; lane 3, *Ovis aries* DNA; lane 4, *Bos taurus* DNA; lane 5, *Gallus gallus* DNA; lane 6, *Equus caballus* DNA; lane 7, *Equus asinus* DNA; M, 100-bp ladder (100 to 1000 bp).

gene amplification. This approach was less time-consuming than the one previously described by Matsunga et al. (1999).

Polymerase Chain Reaction

The CB7u and CB7l primer produced specific fragments of about 195 bp for ruminant species. The primer specificity for identification of the extracted DNA from species is indicated in Figure 1. 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 DNAs identification and detection in food materials 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; Ghovvati et al., 2008). The primer binding sites were chosen so that it can generate specific amplimers less than 200 bp. Furthermore, it is feasible to present a method capable of identifying animal species DNAs in food materials which have undergone severe preparation processes. This degradation can, however, cause problems in using PCR method that have been reported by Bottero et al. (2003).

Testing samples

The applicability of this method in industrial food materials (Kebab loghmeh, minced meat, beef burgers and canned stew) has been proved by this study. The results are shown in Figure 2. Results of RFLP assay reveal that 6/7 (86%) of Kebab loghmeh samples, 6/10 (60%) of minced meat samples and 2/5 (40%) of canned stew samples were contaminated by sheep residuals, while 1/10 (10%) of minced meat samples and 1/8

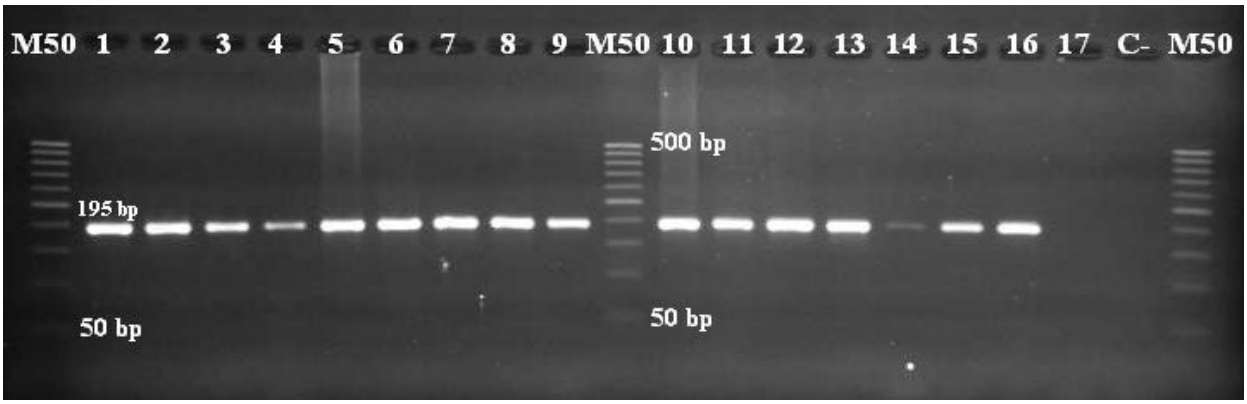

Figure 2. PCR amplification in commercial industrial meat product samples (Kebab loghmeh, minced meat, beef burger and stew conserve). Lane 1, Positive control; lane 2, beef burger (A); lane 3, beef burger (B); lane 4, beef burger (C); lane 5, beef burger (E); lane 6, minced meat (A); lane 7, minced meat (B); lane 8, minced meat (C); lane 9, minced meat (D); lane 10, canned stew (A); lane 11, canned stew (E); lane 12, canned stew (B); lane 13, Kebab loghmeh (A); lane 14, Kebab loghmeh (B); lane 15, Kebab loghmeh (G); lane 16, Kebab loghmeh (C); lane 17, Kebab loghmeh (D); C-, negative control reagent; M, 50-bp ladder (50 to 500 bp)

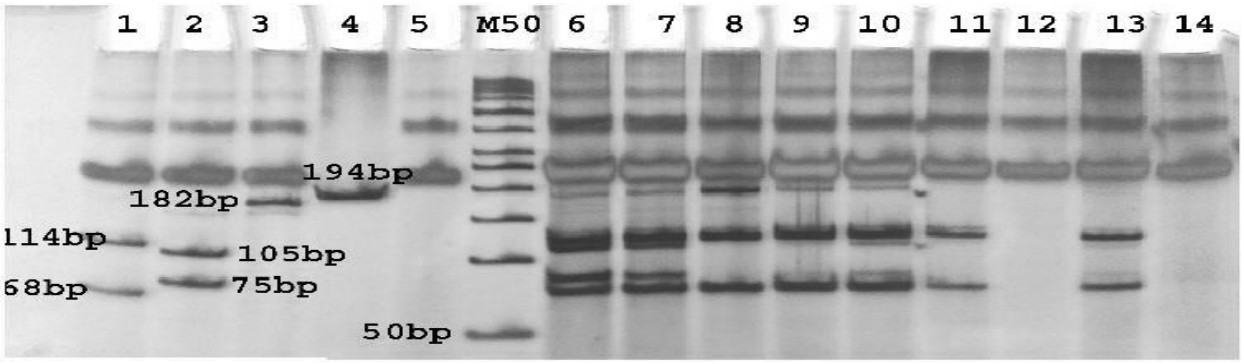

Figure 3. Restriction profiles of ~195 bp Cyt-b PCR products obtained after treatment with *Sse*9I on 8.0% polyacrylamide gel. Lane 1, *Bos taurus*; lane 2, *Ovis aries*; lane 3, *Capra hircus*; lane 4, positive control (undigested); lane 5, negative control reagent; lane 6, minced meat (A); lane 7, minced meat (B); lane 8, Kebab loghmeh (F); lane 9, beef burger (A); lane 10, beef burger (B); lane 11, canned stew (C); lane 12, beef burger (E); lane 13, Kebab loghmeh (A); lane 14, canned stew (E); M50, 50-bp ladder (50 to 500 bp).

(12.5%) of beef burgers samples were contaminated by goat residuals in contrast to what was mentioned on their labels. Cross-contamination of goat and sheep residuals in Kebab loghmeh, minced meat and beef burgers were 14, 20 and 50%, respectively (Figure 3). In total, 80% of the collected samples were contaminated by prohibited ruminant residuals in contrast to what was labelled. With respect to all Iranian food producers, our results indicate that only 20% of the food labels were approved by DNA test. Fraud in meat based products and their side products by adding low quality and cheap meat has always been an issue in food industries (Girish et al., 2005; Zha et al., 2010).

The PCR-RFLP method used in this research is very specific to be applied for food products that have endured severe heating condition during preparation process. Moreover, the PCR-RFLP is a cost-effective and reliable method for samples which have been deformed and contain only scanty amounts of DNAs from various species. This method also plays an important role in the food industry regulation and legal issues. Direct sequence determination method for testing the samples is highly reliable and accurate; however, it is costly and time consuming (Meyer et al., 1995; Partis et al., 2000). The PCR-RFLP method as an alternative to direct sequence determination of PCR products, is an efficient technique for identification of animal species used in food materials (Fajardo et al., 2006; Pfeiffer et al., 2004; Rea et al., 2009). During judicial enquiries, limited amount of samples and also usually damaged samples during the production process, rRNA and mtDNA are generally used for fraud identification and species identification. This is due to the fact that these genes have more copies per each cell in these regions compared to genomic DNA,

and this distinction is statistically significant.

Consumer groups and food producers themselves are increasingly demanding regulations for food safety. In Iran, food products are controlled by established rigid laws for labeling of products and all industry managers are familiar with the laws. Therefore, the information claimed on the food products labels must exactly reflect the products' content. To control the quality of the food products, it is required to develop new methods capable of proving and detecting various species used in them (Ghovvati et al., 2009). A recent study by Doosti et al. (2011) reported a detected adulteration in industrial meat products by PCR-RFLP assay in Iranian commercial meat products. Their result indicate that 6 of 68 fermented sausages (8.82%), 4 of 48 frankfurters (8.33%), 4 of 55 hamburgers (7.27%), 2 of 33 hams (6.6%), and 1 of 20 cold cut meat (5%) were contaminated with prohibited meat. In addition, Chen et al. (2010) developed an efficient PCR-RFLP method for authenticating meat products from five farm animals on the basis of the mitochondrial 12S rRNA gene sequence variations. They successfully detected cattle DNA in yak DNA at ratios of 2:8 to 5:5 in one reaction, using this method. Di Pinto et al. (2005) also developed duplex PCR for detection of pork meat in horse meat fresh sausages. Their results reveal the presence of pork meat in 6/30 and the total absence of horse meat in 1/30 of the analyzed horse sausage samples. In this research, one universal primer pair along with one endonuclease enzyme was used for simultaneous identification of three ruminant species, while some studies used several different primers followed by enzymatic digestion (Myers et al., 2003; Santaclara et al., 2007; Stamoulis et al., 2010).

Conclusion

The correlation between the obtained results and determined presumptions demonstrate that the PCR-RFLP is a powerful method not only for genetic research and identification of different types of animal species based on mitochondrial DNA sequence, but can also be a powerful, reproducible and rapid test for detection and separation of ruminant species residuals in commercial meat products even in cases of mixtures of species, and thus can be widely used in diagnostic laboratories. PCR-RFLP is a cost-effective and reliable method for heated and processed food products containing low concentrations of DNA. The PCR-RFLP method described herein was optimized and used to monitor and control the quality of food products and identified frauds in some of the collected samples. The results indicate the necessity of PCR-based tests in Iranian national standards to increase the quality of meat products, customers' health and satisfaction.

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