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Identification of bovine, ovine and caprine pure and binary mixtures of raw and heat processed meats using species specific size markers targeting mitochondrial genome

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Summary

A specific polymerase chain reaction (PCR) method was applied for identification of bovine (*Bos taurus*), ovine (*Ovis aries*) and caprine (*Capra hircus*) pure and binary mixtures of raw and heat-processed meats. These meats are used in food industry products and/or for direct consumption of consumers. The mitochondrial DNA was amplified as a template in a PCR reaction by use of specific primers related to each species. Specific primers with mitochondrial origin amplified amplicons with the length of 300, 172 and 122 bp in target regions in cattle, sheep and goat, respectively. For determination of the primer sensitivity a set of binary meat mixtures with 0.1, 0.5, 1, 5, 10, 50 and 100% based on weight ratio was tested. The detection limit was found to be 0.1% for all samples. In heat-processing program boiled water for 20 min, 121°C for 20 and 30 min and 127°C for 20 min by autoclaving was used, similar to the conditions that are used in pasteurization, sterilization and also meat processing in industrial factories. The performance of the method was not affected by prolonged heat treatments. The results obtained in the present study confirmed the efficiency of species specific primers for targeting of mitochondrial genome in order to detect bovine, ovine and caprine pure and binary mixtures of raw and heat processed meats with high sensitivity and accuracy.

Key words: Species specific primers, Meat mixtures, mtDNA, Food traceability

Introduction

The adulteration or substitution of meat has always been a source of concern for various reasons such as public health, religious matters, and unhealthy competition in meat market, and also, consumers have become more conscious about the source of food and expect the source of food to be authentic (Kumar *et al*., 2011). Mis-representation of a costlier meat with a cheaper one is one of the most common examples of fraudulence prevalent in meat industry (Girish *et al*., 2004). For prevention of this type of fraud, food labeling regulation requires the identification of meat origin, because animal meats often undergo a tremendous amount of processing before being sold. Species of meat present in processed, cooked or compounded mixtures are not always possible to identify by routine examination, and hence different analytical methods have been developed for meat species identification and to authenticate that meat is genuine and acceptable to the consumers (Haunshi *et al*., 2009). Several analytical approaches have been taken for meat species identification in a wide array of degraded and processed substrates, broadly based on detecting either protein or DNA (Aida *et al*., 2005). Protein based methods include immunoassays, which are the most widely used, with several companies supplying kits for a range of species. However, because proteins are denatured during heat and pressure processing, only antibodies raised against heat-stable biomarkers can be used for detection of species in processed samples (Hossner *et al*., 2006). For this reason in addition to the high

cost, immunological analyses have been replaced by DNA-based methods (De *et al*., 2011). DNA has the advantage of being a relatively stable molecule, is more able to withstand heat and pressure processing and also, its structure is conserved within all tissue of an individual (Lanzilao *et al*., 2005). There are numerous nuclear DNA based procedures available to identify precisely the origin of meat species such as DNA hybridization (Chikuni *et al*., 1990), cloth based hybridization array system (Murphy *et al*., 2007) random amplified polymorphic-DNA polymerase chain reaction (RAPD-PCR) fingerprints (Koh *et al*., 1998; Calvo *et al*., 2001), species specific satellites DNA probes (Buntjer *et al*., 1998), PCR-RFLP, DNA sequencing and species specific PCR (Nagappa, 2008; Sahilah *et al*., 2011).

Although earlier methods proved to be accurate and useful in identification of species origin of meat, they suffer from certain disadvantages. While reproducibility of RAPD-PCR is a matter of concern due to the requirement of high stringent conditions (Koh *et al*., 1998), sequencing of genes and PCR-RFLP procedures are expensive, requires more time and more analytical work and interpretation of the results is somewhat complex (Ballin *et al*., 2009). Further, PCR-RFLP in admixture meat and meat products makes the procedure unfeasible (Ilhak and Arsalan, 2007). Considering the above-mentioned facts, the objective of the present study was the identification of bovine, ovine and caprine in pure and binary mixtures of raw and heat-processed meats by PCR.

Materials and Methods

Collection of samples

Fresh meat samples of bovine (*Bos taurus*), ovine (*Ovis aries*) and caprine (*Capra hircus*) leg were collected from local butcheries and slaughterhouse. After collection, they were transported to the laboratory under refrigeration and processed immediately or stored at -20°C until used. Meat samples were also analysed after being subjected to boiling water and sterilization (121°C for 20 and 30 min, and 127°C for 20 min) treatments. In order to evaluate the test sensitivity, binary mixtures of bovine, ovine and caprine muscles were prepared (100, 50, 10, 5, 1, 0.5 and 0.1% (w/w)).

DNA extraction

DNA was extracted by a modified high salting-out method. This method is cheap, non-phenolic and non-toxic. 500 mg of each sample was used for DNA extraction. Briefly, all the specimens were subjected to enzymatic digestion by addition of 600 µl of TNES buffer (10 mMTris, 400 mM NaCl, 100 mM EDTA and 0.6% SDS (pH = 7.5)) and 30 µl of Proteinase-K (Qiagen, Germany, 20 mg/ml) and the samples were incubated overnight (or 5-24 h) at 50°C. The above digested materials were processed for DNA isolation using high salting-out method. After incubation, 167 µl of 6 M NaCl was added and shaken vigorously for 20 s. Then, samples were centrifuged 13000 rmp for 9 min at room temperature. Supernatant was removed to a new tube and 800 µl of cold 100% ethanol was added. After several inversions, DNA was precipitated in solution. The sample was then centrifuged at 13000 rmp for 15 min at room temperature, supernatant was removed and the pellet washed in 500 µl of 100% ethanol. After pouring the ethanol the DNA pellet was washed again with 70% ethanol. After removing the 70% ethanol, the sample was left to air dry. The DNA was then resuspended in 180 µl of sterile distilled water or Tris-EDTA.

Polymerase chain reaction

The set of primers used for amplification are listed in Table 1. These primers were applied for the first time for detection and identification of meats origins. Species specific primers were designed from different regions of Mitochondrial D-loop, 12S-16S rRNA and 12S rRNA for bovine, ovine and caprine, respectively (Bottero *et al*., 2003; Lopeza-Calleja *et al*., 2005; Kotowicz *et al*., 2007) (Table 1).

Polymerase chain reaction amplification reactions were performed in a total volume of 25 µl. Each reaction mixture contained 100 ng of template DNA, 1.5 mM $MgCl_2$, 2.5 µl 10 X PCR buffer (100 mM Tris-HCl, 500 µl mM KCl, pH = 9.0), 0.5 of each of

Table 1: The primers sequence used in polymerase chain reaction

Species	Primer sequence (5′-3′)	Gene	Size of amplicon
Bovine	F:5′-CAATAACTCAACACAGAATTTGC-3′ R:5′-CGTGATCTAATGGTAAGGAATA-3′	D-loop	300 bp
Ovine	F:5′-ATATCAACCACACGAGAGGAGAC-3′ R:5′-TAAACTGGAGAGTGGGAGAT-3′	12s-16s rRNA	172 bp
Caprine	F:5′-AAACGTGTTAAAGCACTACATC-3′ R:5′-GTCTTAGCTATAGTGTATCAGCTGCA-3′	12s rRNA	122 bp

the forward and reverse primers, 0.2 µl smartTaq, 0.2 µl dNTP and distilled water. PCR amplification was carried out in a Master Cycler Gradient Thermo cycler (Eppendrof, Germany). For cattle species specific primers: thirty-five cycles at 95°C for 30 s, 52°C for 30 s and 72°C for 2 min were performed. For sheep species specific primers: after an initial denaturation step at 94°C for 5 min, 35 cycles were programmed as follows: 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, final extension at 72°C for 5 min. For goat species specific primer: an initial heat denaturation step at 93°C for 2 min, followed by 35 cycles consisting of 93°C for 30 s for DNA denaturation, 63°C for 30 s for primers annealing, and 72°C for 45 s for DNA extension. The last extension step at 72°C was maintained for 3 min.

Gel electrophoresis

PCR products were electrophoresed through 2-2.5% agarose gel for 85 V at room temperature. The obtained bands were visualized by ethidium bromide staining and UVP transilluminator (Bio-Rad Laboratories INC).

Results

Species specific primers and optimum PCR conditions were applied for detection of cattle, goat and sheep tissues in pure, mixed and heat-processed meats. The primers were set to amplify species specific fragments of 300, 172 and 122 bp in length for cattle, sheep and goat, respectively. The specificity of each primer was confirmed by PCR amplification of bovine, ovine, goat, duck and chicken genomic DNA. However, no cross-reactivity among different species was observed.

Sensitivity of the species specific primers

To determine the detection limit of the assay, PCR amplification was performed on muscle binary mixtures containing 0.1, 0.5, 1, 5, 10 and 50% (w/w) of the target species in mixed meats. The price of these meats and their products are different. However, meats from these species are difficult to identify by morphological characters and sensory differences except for specifically trained people. Consequently, there is a need for the development of analytical methods to detect mislabeling or fraudulent substitution of the most expensive meat products with cheaper ones. The sensitivity of the method was determined as meat mixes at 0.1% level for each species and the results were observed on agarose gels (Figs. 1-3).

Satisfactory PCR results were also accomplished when the effect of thermal treatment was examined. The identification of species specific size marker was studied through PCR analysis of DNA extracted from meat that were treated in boiled water for 20 min, 121°C for 20 and 30 min

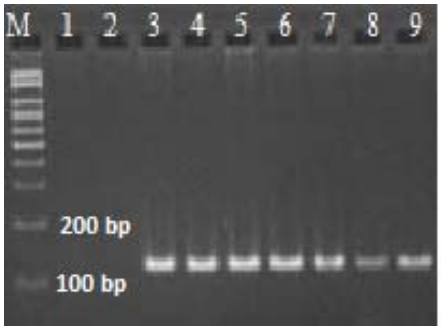

Fig. 1: Electrophoresis of goat species specific primers. (1) 100% cow, (2) 100% sheep, (3) 100% goat, (4) 50% + 50% goat and sheep, (5) 10% + 90% goat and sheep, (6) 5% + 95% goat and sheep, (7) 1% + 99% goat and sheep (8) 0.5% + 99.5% goat and sheep, (9) 0.1% + 99.9% goat and sheep. M: is 100 bp molecular marker

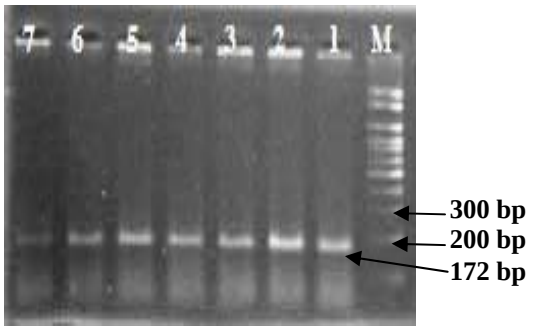

Fig. 2: Electrophoresis of sheep species specific primers. (1) 100% sheep, (2) 50% + 50% sheep and goat, (3) 10% + 90% sheep and goat, (4) 5% + 95% sheep and goat, (5) 1% + 99% sheep and goat, (6) 0.5% + 99.5% sheep and goat, (7) 0.1% + 99.9% sheep and goat. M: is 100 bp molecular marker

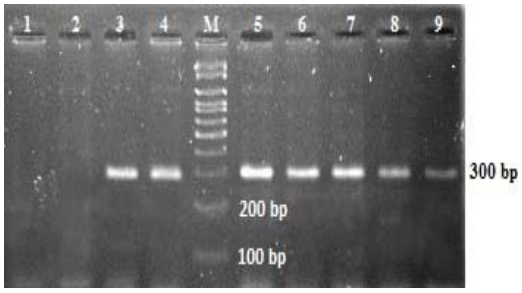

Fig. 3: Electrophoresis of cattle species specific primers. (1) 100% sheep, (2) 100% goat, (3) 100% Cow, (4) 50% + 50% cow and sheep, (5) 10% + 90% cow and sheep, (6) 5% + 95% cow and sheep, (7) 1% + 99% cow and sheep, (8) 0.5% + 99.5% cow and sheep, (9) 0.1% + 99.9% cow and sheep. M: is 100 bp molecular marker

(sterilization) and 127°C for 20 min in autoclave conditions. These temperatures are commonly applied for meat processing in food industries and manufacturing.

Discussion

To protect consumers from falsely labeled, low quality meat products, various techniques have been reported for species identification in an effort to prevent such fraudulent actions (Herman, 2001). In recent years, DNA based methods have been widely used in identification of meat products (Lenstra *et al*., 2001). Among them, PCR in particular, has proven extremely useful in tracing the species origin in food (Colagen *et al*., 2001). In this work, species specific size markers were used on the mitochondrial DNA sequences to attempt PCR identification of meats from the bovine (*Bos taurus*), ovine (*Ovis aries*) and caprine (*Capra hircus*) origin. Traceability issues have grown in importance due to consumers' increasing attention to food quality matters. Consumers are particularly worried about meat quality and its origin and integrity all through the food chain until consumption. In this sense, the European Union has applied strict legislation on labeling to guarantee meat traceability. Regulation by the EC legislation (178/2002) on food traceability (European Commission, 2002) requires all stakeholders within the food supply chain to be able to identify the source of all raw materials. Therefore, to achieve this aim, a quick, precise, simple and cheap method should be applied for detection of the lowest levels of adulterations to lessen consumers worries. The protein and DNA based technologies can be used in food control laboratories to carry out species differentiation of raw materials to be used for industrial food preparation and the detection of animal species in food products. DNA techniques have become very important and are widely used because DNA is a relatively stable molecule allowing analysis of processed and heat treated food products. In this study, species specific primers were used to identify raw and heat processed meats from three species including sheep, cattle and goat. The results of the present study confirmed the usefulness of species specific size markers based on mitochondrial genome for detection of adulteration in raw and heat processed meat mixtures of farm animals by lowest detection limits. Bottero *et al*. (2003) designed specific primers for mitochondrial 12s and 16s rRNA genes to identify cows', goats' and sheep's milk in dairy products. The multiplex PCR generate fragments of different length for each species. They could detect goat, sheep and cows' milk in dairy products with a good sensitivity threshold (0.5%). In another study, the use of specific primers pairs for quail, pheasant, partridge and guinea fowl allowed the selective amplification of the desired avian sequences of the mitochondrial 12S rRNA gene by PCR. The authors suggested the assay is

sensitive and specific and can be useful for the accurate identification of meats from game bird species, avoiding mislabeling or fraudulent species substitution in meat products (Rojas *et al*., 2009). In the present study, there was no significant influence between heat processing effects and the presence of desired bands in PCR products for detection and identification of processed meats. Moreover, this assay has shown the performance of the method was not affected by prolonged heat treatments. In agreement with our results, Matsunaga *et al*. (1999) and Rojas *et al*. (2009) have confirmed the ability of the PCR to amplify relatively short segments in heat processed samples with highly damaged DNA. In our study, the sensitivity of 0.1% was determined for cow, goat and sheep pure and binary mixtures of raw and heat processed meats that are commonly used in food industry and retailers. This detection limit was confirmed by Martin *et al*. (2009). They reported the detection of porcine DNA in mixtures containing as little as 0.1%. Compared to alternative techniques for species identification such as PCR-RFLP (Pascoal *et al*., 2004), DNA sequencing (Colombo *et al*., 2002) or real-time PCR (Hird *et al*., 2005), PCR using specific primers offers the advantages of being cheaper and more useful for routine analysis of large numbers of samples.

In conclusion, this study confirms the usefulness of PCR for identification of pure and binary mixtures of raw and heat processed meats because of its simplicity, quickness and cost-effectiveness of species specific size markers targeting mitochondrial genome, and it is precise. Therefore, for consumer awareness and also for unhealthy competition in meat market, this assay might be used by the National Food Safety Authorities Departments for evaluation of meat and meat products.

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