Identification of Animal Species in Meat Broth by Simplex and Multiplex PCR

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Abstract:

In some country, some restaurants may use fraudulent substitution in meat product that has less commercial value or undesirable. Effective laboratory tests must be available for prevention of this phenomenon. In this study meat broth sample was successfully used to identify seven animal species in cooked meals by simplex and multiplex PCR using species specific primers for conserved region of mitochondrial DNA (mtDNA). The results showed effective amplification of mtDNA without cross reaction between primers. Whereas existing methods require mincing and homogenization of direct meat samples, this meat broth sampling method can be performed directly and is thus easier to perform. In conclusion, meat broth sample could be used instead of direct meat tissues for species identification of cooked meat in restaurants to control adulteration substitutions of undesirable meat and protection of the customer rights

Keywords: Meat broth sample; species specific primer; veterinary forensic medicine; meat identification

Introduction

Many meat products contain several species mixed together in different proportions and undetectable by either the naked eye or by tasting (1). Unfortunately, this has enabled meat adulteration to become a common practice in some countries with some restaurants fraudulent substituting meats for product that have less commercial value or undesirable. Therefore, identification of the species present in meat products has become important in order to protect consumers from illegal and undesirable adulteration on religious, economic and health grounds (2,3) .

Existing methods for detecting the species composition of raw meat includes sensory analysis, anatomical (4) histological and immunological differentiation, however all of these methods have limitations (5). The main problem is that these methods depend on protein analysis and these proteins may be denatured on processing and heating, leading to subsequent loss of analytical specificity (1). Therefore, species determination of ground meat or mixed meat product after cooking may be not reliable.

Biomolecular approaches can avoid these problems. Some techniques applied in the past for meat species identification include DNA hybridization, Random Amplified Polymorphic DNA-PCR (RAPD–PCR) and restriction fragment length polymorphism (RFLP) analysis. More recently, molecular

techniques such as species-specific real-time PCR, simplex and multiplex PCR have received particular attention (2).

Mitochondrial genes are particularly suited for species detection by PCR assay. Because each mitochondrion has between 2 and 10 copies of mitochondrial DNA (mtDNA), and each cell can have up to 1000 mitochondria, mtDNA is present in cells at much higher copy number than nuclear DNA. This property significantly increases the sensitivity of molecular testing (6). Mitochondrial genes such as cytochrome b gene, D-LOOP gene, 12S rRNA and 16S rRNA have previously been demonstrated to be effective for species identification in food products (2).

In this study, meat broth sample was used instead of direct meat tissues for species identification and monitoring of cooked food in the suspected restaurants.

II. Materials and methods :

A. Sample collections

The ruminant and chicken meat samples were purchased from local butchers. The pork sample was obtained from veterinary department of Sulaimani international airport. Cat and donkey samples obtained from the veterinary college of Sulaimani university. The upper phase of meat broths was randomly sampled from 3 restaurants: Aqary, Muhammad and Darya in Sulaimani city.

B. Preparation of meat broth

Meat broth was prepared in the laboratory by cooking contemporarily meat from different species in one broth. Firstly, 5 grams of intact meat sample were mixed from each of 7 animal species; cow, goat, sheep, cat, donkey, pig and chicken. 250ml of water then was added to the mixture of meat with a pinch of salt and cooked on benzene burner, until the meat samples were fried with water. Then 2 litters of water were added to the meat and it was boiled for a further 90 minutes.

C. DNA extraction

DNA was extracted from the laboratory samples and the samples obtained from the three restaurants by the following; 10 ml of meat broth was centrifuged at 4000 g for 5 minutes, the supernatant discarded and DNA extracted from the pellet using a Bioneer Accuprep®Genomic DNA extraction kit according to the manufacturer's instruction. Extracted DNA samples were labeled and preserved at -20°C for subsequent PCR assay.

D. Primers

Species-specific primers for detection of feline were designed by Ilhak and Arsalan (5) as in Table 1. Primers for ovine, caprine, and bovine were taken from Matsunaga et al. (7) and Lahiff et al. (8). Primers for swine, poultry and farm ruminants were designed by Dalmasso et al. (9) and the specific primer for donkey was designed by Kesmen, Sahin, and Yetim,(10). All primers were synthesised by Bioneer.

E. PCR amplification

The PCR amplification reaction was carried out in 0.2 ml tubes using Bioneer AccuPower PCR PreMix. Simplex PCR reaction was performed with 5µl of DNA template and 3µl of 10 pmol of reverse and forward specific primers according to

Table 1 : species specific primers for used in specific PCR identification

Species	Direction	Primer	Amplicon size (bp)	Genes
Donkey	foreword	CATCCTACTAACTATAGCCGTGCTA	145	ND2
	Reverse	CAGTGTTGGGTTGTACACTAAGATG		
Poultry	foreword	TGAGAACTACGAGCACAAAC	183	12S RNA
	Reverse	GGGCTATTGAGCTCACTGTT		
Pig	foreword	CTACATAAGAATATCCACCACA	290	12S RNA-tRNA Val
	Reverse	ACATTGTGGGATCTTCTAGGT		
Ruminant	foreword	GAAAGGACAAGAGAAATAAGG 3	104	16S RNA
	Reverse	TAGGCCCTTTTCTAGGGCA		
Bovine	foreword	GCCATATACTCTCCTTGGTGACA	271	Cytochrome b
	Reverse	GTAGGCTTGGGAATAGTACGA		
Sheep	foreword	TTAAAGACTGAGAGCATGATA	225	Cytochrome b
	Reverse	ATGAAAGAGGCAAATAGATTTTCG		
Cat	foreword	CATGCCTATCGAAACCTAACATAA	274	ND4
	Reverse	AAAGAAGCTGCAGGAGAGTGAGT		
Goat	foreword	GACCTCCCAGCTCCATCAAACATCT CATCTTGATGAAA	157	Cytochrome b
	Reverse	CTCGACAAATGTGAGTTACAGAGG GA		

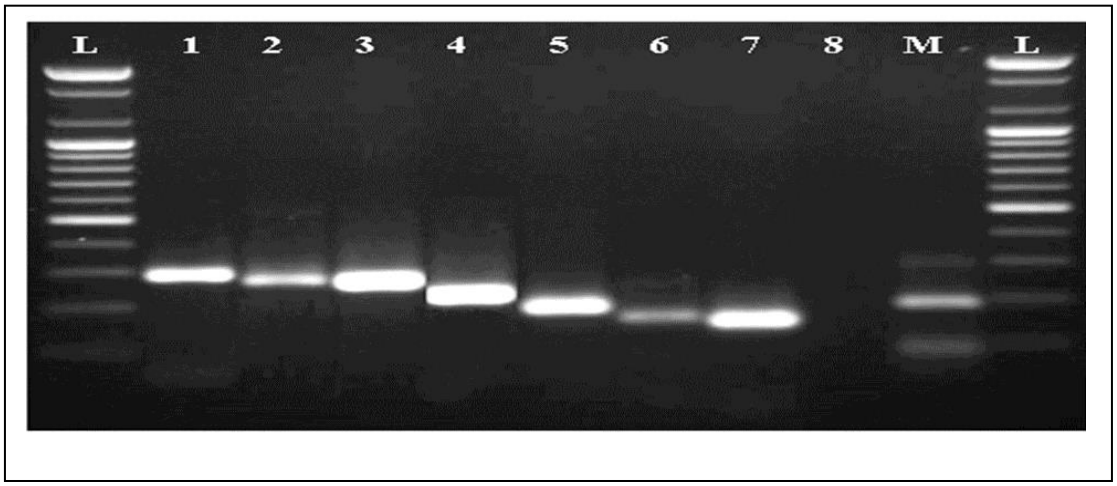

Fig. (1) Electrophoretic gel analyses of laboratory prepared meat broth consist of seven animal species. L: DNA ladder (100 bp), 1: 290 bp pig, 2: 274 bp cat, 3: 271 bp bovine, 4: 225 bp sheep, 5: 184 bp chicken, 6: 157 bp goat, 7: 145 bp donkey, 8: Negative control, 9: multiplex PCR showed 104 bp, 184 bp and 290 bp for ruminant, chicken and pig, respectively.

animal species (Table 1) and made up to a final volume of 50µl with dH2O. Multiplex PCR reaction was performed using 5µl of DNA template with 3µl of 10 pmol of reverse and forward primers of each of ruminant, poultry and pig in a final reaction volume of 50µl. The reaction was carried out in a Thermocycler (Techne, UK). The primers have different melting temperature; therefore the annealing temperature was optimized first, based on mixture of DNA which was extracted separately from uncooked meat samples from the seven animals. The best annealing temperature was 60 °C. Secondly, the mixtures of meat broth sample were subjected to the same optimized annealing temperature. The thermocycler programmed for an initial denaturation at 94°C for 4 mints ; 35 cycles consisting of denaturation at 94°C for 30s, annealing at 60°C for 15s, and extension at 72°C for 30s; and then a final extension phase run at 72°C for 5 minutes. Electrophoresis was performed by running 10µl of PCR product on an agarose gel (1% w/v) at 92V for 1 h. The gel was stained with ethidium bromide (0.4 µg/ml), visualized using a UV transilluminator and photographed.

III. Result :

The mtDNA of each species was successfully amplified by using primers specific PCR assay for each animal species. In simplex PCR; the primers generated specific fragments consisting of 145 bp for donkey, 157 bp for goat, 184 bp for chicken, 225 bp for sheep, 271 bp for bovine, 274 bp for cat and 290 bp for pig. The electrophoresis pattern indicate single specific band with expected size and absence of cross-contamination. Laboratory prepared meat broth was also analysed by multiplex PCR using primer for ruminant, chicken and pig. The gel showed bands at 104 bp, 184 bp and 290 bp, respectively. The primer mixture selectively amplified mtDNA of each meat species and there were no unspecific bands.

The meat broth from restaurants was subjected to species identification. Aqary restaurant showed mtDNA amplification of bovine, sheep and goat. Both Darya and Muhammad restaurant showed mtDNA amplification of chicken only (data not

shown). These results confirmed the chefs' statements.

VI. Discussion :

Species identification of cooked meat is important for controlling fraud in the catering industry. In poor countries and countries which are suffering from internal disturbances, substitution of meat product may occur with cheaper meat like street animals and forbidden meat. Therefore, identification of meat species present in food is crucial in veterinary forensic medicine.

Humans are not capable of reliably recognizing mixed meats after cooking using their senses alone (1). Some molecular methods for species identification are expensive like Real time PCR (10), whilst others are time-consuming and complex like DNA hybridization. However, antigen-antibody reaction techniques such as ELISA and immunoelectrophoresis, immunodiffusion do not provide reliable results (11,12). Conventional PCR assay can play an important role in rapid and sensitive identification of animal species (3).

Prior works in this area has generally relied on meat tissue or processed mixed meat for identification of animal species (1, 2, 3). In this study, however, meat broth from a cooked meal was used for
identification of animal species and our results showed effective identification of mtDNA in all animal species. Furthermore, the results confirmed that there was adequate amount of target DNA for PCR amplification in a meat broth. This affirms the advantages of using high copy number mtDNA over nuclear DNA (6). mtDNA separated from the cooked tissue and released into the broth provided enough target DNA for amplification and produced an intense gel electrophoresis band after staining with ethidium bromide.

Sampling of meat broth was easier and more representative for testing meals containing a mixture of different animal tissues. Moreover, meat broth sampling avoids the potential sources of contamination resulting from chopping, mincing and homogenizing meat samples in direct meat sample methods (13,10) . Therefore meat broth sampling may be more reliable.

The primers chosen were selected to amplify short fragments of mtDNA to minimize the effects of DNA degradation due to high cooking temperatures (13).

There was no cross-reaction between the primers for different species. This was possible because the species - specific primers were selected on the conserved regions within the mtDNA according to each animal species (2).

V. Conclusion:

This article demonstrates that meat broth sampling with simplex and multiplex PCR is a promising method for identifying the animal species present in a cooked meal. Furthermore, it is suggested that meat broth is a representative and easily applicable method for detecting a mixture of different animal tissues in cooked meal. Therefore, it can be used for identification of fraudulent substitutions of undesirable meat and protection of the customer rights.

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