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Research Article

Differentiation of Adulterated Meat Products through Molecular Technique: PCR-RFLP

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Abstract: Meat adulterations of different species are undetectable and it is common practice globally. In the field of food analysis, species determination is mostly sufficient, but simultaneous detection of several species in a single food product is desirable. The aim of the study was to distinguish between meats of two different species through PCR-RFLP analysis. The meat of two species were used include domestic pig (*Sus scrofa;* Porcidae) and domestic goat (*Capra hircus;* Bovidae). DNA was isolated from these samples , followed by amplification through PCR and further species was differentiated by RFLP using five different restriction endonuclease (RE) enzymes. The DNA sequences of different species are different, hence does not digest by same enzyme. The number and position of bands obtained after digestion were different in two species. In case of meat adulteration, the specific number and position of bands of DNA of a particular species will not be obtained, rather bands will be formed at intermediate positions and number of bands may vary. Thus, PCR-RFLP method is a potential tool for forensic identification and to differentiate specific meat sample and this molecular technique is an important tool to examine adulteration in meat food products.

Key words: Differentiation, DNA, Meat, PCR, Restriction enzyme, RFLP

INTRODUCTION

Molecular techniques developed over the last two decades helped in developing authentic and reliable methods for species identification from meat. Many meat products now a day may contain several species in different proportions mixed together and undetectable by naked eyes or by eating. Meat adulteration has become a common practice in most countries. In the field of food analysis, species determination is mostly sufficient, but simultaneous detection of several species in a single food product is desirable.

Developments in molecular biology have facilitated identification of plant, bacteria and animal species with high accuracy (Aguado *et al*., 2001; Weder *et al*.,2001). Polymerase chain reaction (PCR), Restriction fragment length polymorphism (RFLP) and Random amplified polymorphic DNA (RAPD) techniques have been frequently used for identification of meat species (Meyer *et al.,* 1996; Alves *et al.,* 2002). Reason is that nucleic acid based analysis is becoming more and more popular for identification and differentiation of food and food products (Meyer *et al* 1995).

In this study, aim is to distinguish meat of different species through PCR-RFLP method. This is achieved by isolating DNA, from meat sample of two species namely, domestic goat (*Capra hircus*) and domestic pig (*Sus scrofa*), amplifying the isolated DNA using cytochrome b and porcine primers and RFLP digestion using appropriate restriction enzymes which help in identification of meat samples and differentiation of different species and examining adulteration in meat food products.

MATERIALS AND METHODS

Sample: Meat of domestic pig and goat were chosen and obtained from the market in Dehradun (U.K.). These were the muscle tissue samples collected in a collection bottle containing silica granules and were placed at -20˚C before the extraction of DNA in order to prevent sample spoilage and degradation of DNA.

Extraction of Genomic DNA: Six meat samples (goat n=3 and pig n=3) were used. 0.25gm of each meat sample was sliced using sterile dissecting scissors and forceps and were labeled as P1, P2, P3 (pig sample) and G1, G2, G3 (goat sample). DNA was extracted from the meat sample using Koh *et al.,* (1998) method with slight modification which involves four basic steps:1- Digestion 2- Phenol:Chloroform:Isoamyl (PCI) treatment 3- Washing of DNA 4-Solubilizing DNA in T.E. Quantification of total DNA was done by using Spectrophotometer at A260/280 and purity index was checked by Biophotometer.

Amplification of DNA (PCR): Universal primers (Cytochrome b) and the species-specific primer (Porcine) were used (Table 1). The final volume for the PCR reaction for the amplification of DNA was 25µl containing 5µl extracted DNA, 1X Taq buffer, 2.5 mM dNTP, 25mM Mgcl$_2$, 1X BSA and 0.5 µl of each primer and 1U/ µl of Taq DNA polymerase. PCR reaction condition for Cyt b primer was set as follow: 1-Hot start at 94˚C for 2 minutes, 2- Denaturation at 94˚C for 45 seconds, 3-Annealing at 56˚C for 60 seconds and 4-Extension at 72˚C for 60 seconds and for Porcine primer: 1-Denaturation at 94˚C for 45 seconds, 2-Annealing at 58˚C for 60 seconds and 3-Extension 72˚c for 90 seconds. Step 1 was conducted for first cycle only, step 2-4 were repeated for 32 cycles in case of Cyt b primer and 35 cycles for porcine primers.

Restriction Endonuclease Digestion (RFLP): The amplified product of different meat samples were subjected to restriction digestion using Alu I, Xho I, Ssp I, Nae I and Hha I restriction endonuclease enzymes (Table 2). 5U of each enzyme were applied to 5µl of amplified DNA in a final volume of 20µl digestion mixture, which contained 1X reaction buffer and sterile water. The digestion mixture was incubated in a water bath for 16 hrs at the specific incubation temperature according to the restriction enzymes used.

The digested samples were electrophoresed through 2% Ethidium bromide stained agarose gel in 1X TAE buffer. The size of the bands produced was compared with 100 bp ladders. Results were estimated by comparing bands of samples and markers bands, which were loaded on the same gel.

RESULT AND DISCUSSION

DNA was successfully extracted from the meat of goat and pig using Koh *et al.,* (1998) method . The quantity, purity index and quality of extracted DNA were examined using a spectrophotometer, biophotometer and gel electrophoresis (Table 3&4). Isolated DNA from pig and goat gave high intensity intact bands on EtBr stained 1% agarose gel (Fig. 1). The bands of high intensity proved that the extracted DNA is sufficient and of good quality to be used for PCR amplification for the amplification of DNA. Two primers namely Cytochrome b (Universal primer) and Porcine (Species specific primer) were used. Bands of the amplified product were noticed in all the samples using Cyt b primer at approximately 388 bp in pig samples and 356 bp in goat samples (Fig. 2) and porcine primer amplified pig DNA samples and bands obtained at 220bp (Fig 3). Amplification status is shown in Table-5.

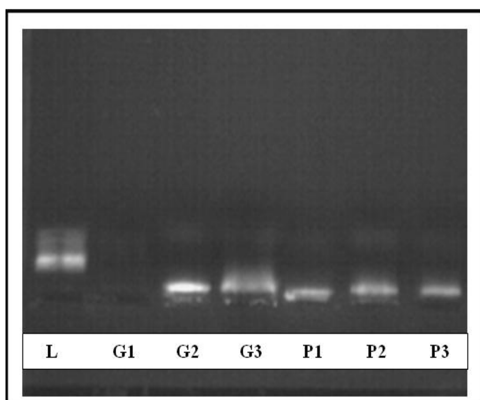

Fig 1. Isolation of DNA from tissue samples (L 100 bp ladder; G1, G2, G3 – Goat and P1, P2, P3 – Pig)

Out of the five restriction enzymes used, pig DNA was successfully digested by Alu I. Single restriction site occur in pig DNA for Alu I, 2 bands of 144 bp and 244 bp were produced (Fig. 4). Single restriction site occur in goat DNA for Ssp I. Ssp I successfully digested the DNA and 2 bands of 196 bp and 160 bp were produced (Fig. 5). The other 3 R.E. viz. Nae I, Xho I and Hha I did not show digestion in any of the sample as there were absence of the recognition sites of these 3, in the amplified DNA of pig and goat meat samples. The result obtained after restriction digestion of samples is given in Table 6 and overall profile is given in Table 7.

Thus, this study helped us to distinguish pig and goat species. Since the goat samples were digested by Alu I and not by any of the other 4 enzymes, whereas SspI digested pig samples. This means that the species differ in their DNA sequences, so the same enzyme did not digest them. Also the position of bands obtained after digestion was different. This indicated that the two samples were of different species and if there in adulteration of the meat sample, the specific number and position of bands of DNA of the particular species is not obtained rather bands are found at intermediate position or a number of bands are observed. Thus, PCR-RFLP is one of the important techniques employed in differentiating the content in food products. The optimized procedure in this study represents a valid PCR-based method to test meat for fast and accurate results. PCR-RFLP employed to discriminate between the two species of meat, allowing detection of falsely declared meat or meat products made up of a single species or mixed samples. The discriminating power of this technique makes it suitable to be used potentially in forensic analysis.

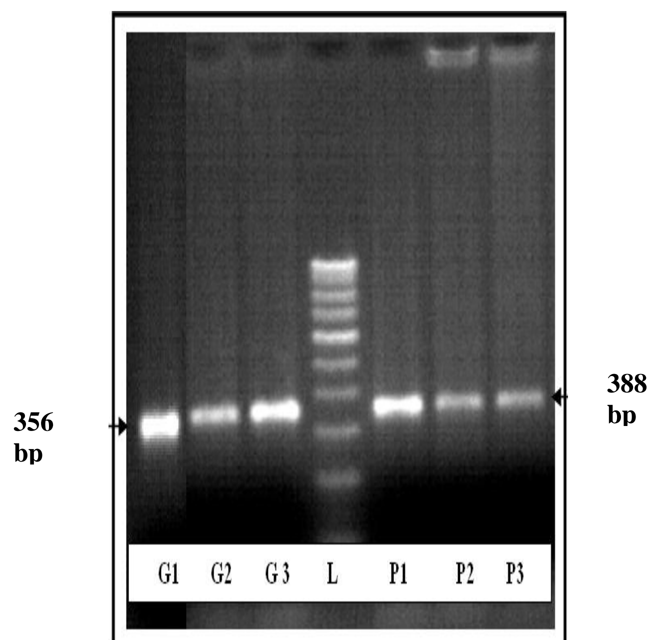

Fig 2. Amplification of isolated DNA using cytochrome b primer. (L 100 bp ladder; G1, G2, G3 – Goat and P1, P2, P3 – Pig)

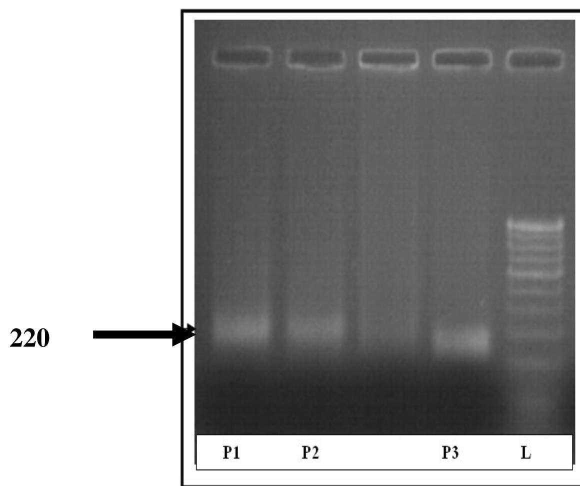

Figure 3. PCR of isolated DNA of pig using porcine primer

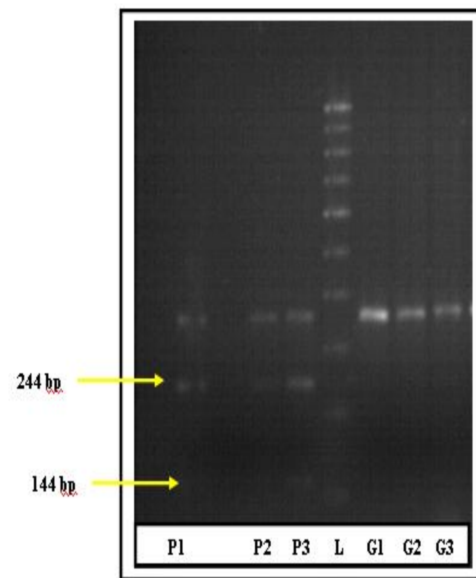

Fig.4. RFLP Using R.E. Alu I

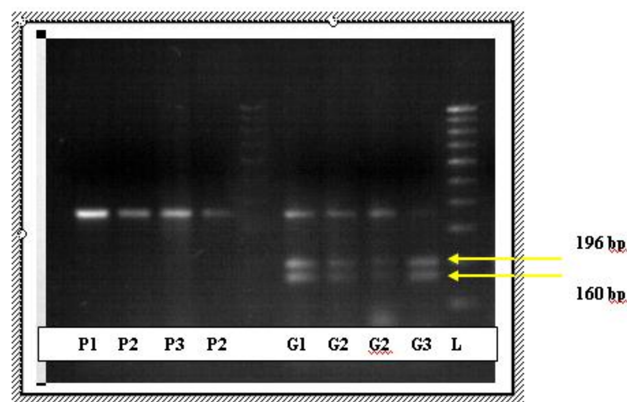

Fig 5. RFLP using R.E. Ssp I

Table 1. List of primers used

Primer	Sequence 5' to 3'	OD	Tm
Cytochrome b –forward	CCATCCAACATCTCAGCATGATGAAA	15.2	63.02
Cytochrome b –reverse	GCCCCTCAGAATGATATTTGTCCTCA	15.58	64.59
Porcine – forward	GCCTAAATCTCCCCTCAATGGTA	29.3	62.77
Porcine – reverse	ATGAAAGAGGCAAATAGATTTTCG	16.29	57.73

OD: Optical Density, Tm: Melting Temperature

Table 2. List of Restriction enzymes used for RFLP analysis

Restriction Enzyme	Recognition site	Temperature	Buffer
AluI	AG↓CT	37^0C	C
HhaI	GCG↓C	37^0C	E+BSA
NaeI	GCC↓GGC	37^0C	E+BSA
SspI	AAT↓ATT	37^0C	B+BSA
XhoI	C↓TCGAG	37^0C	E+BSA

Table 3. Quality of Isolated DNA from Goat and Pig samples

Samples	G1	G2	G3	P1	P2	P3
Very good	-	+	-	+	-	+
Good	-	-	+	-	+	-
Smear	-	-	-	-	-	-
No band	+	-	-	-	-	-

Table 4. Absorbance and Quantity of the isolated DNA samples

S. No.	Sample	Absorbance ($A_{260}/_{280}$)	Quantity of DNA (ng/µl)
I	***Goat***		
1	G1	1.72	121
2	G2	1.65	78
3	G3	1.70	72
II	***Pig***		
4	P1	1.80	80
5	P2	1.83	44
6	P3	1.82	84

Table 5 Amplification status of samples from Goat and Pig samples

Primer	G1	G2	G3	P1	P2	P3
Cytochrome b	+	+	+	+	+	+
Porcine	-	-	-	+	+	+

Table 6 Restriction enzyme actions

Restriction Enzyme	Goat	Pig
AluI	-	+
Hha I	-	-
Nae I	-	-
Ssp I	+	-
Xho I	-	-

Table 7 Number and position of bands after restriction digestion

Restriction enzyme	Pig		Goat	
	No. of band	Position	No. of band	Position
Alu I	2	244, 144	1	356
Hha I	1	388	1	356
Nae I	1	388	1	356
Ssp I	1	388	2	196,160
Xho I	1	388	1	356

CONCLUSION

Mixing in the meat food products cannot be detected by naked eyes or by eating. Detection of several species in a single food product is desirable. We concluded that PCR-RFLP, a nucleic acid based analysis, has found to be an important tool to differentiate the species and to examining the adulteration of different food products. With the help this technique DNA based differentiation can be made as DNA sequence of different species are different and not digested by the same restriction enzyme, so the position and number of DNA bands are found to be different. This becomes the base to differentiate the species using PCR-RFLP technique.

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