MT 12S rRNA GENE PCR-RFLP BASED ASSAY FOR MEAT AUTHENTICATION

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ABSTRACT

For identification and differentiation of meat animal species from known, unknown and adulterated meat samples, the efficacy of mt 12S rRNA gene PCR-RFLP technique on beef, buffalo meat, mutton and chevon was evaluated . All the samples generated 456 bp amplicon on PCR and restriction enzyme digestion of the PCR product with *AluI, HhaI, BspTI* and *ApoI* revealed characteristic RFLP patterns. The method was validated by known samples and the species of origin was identified. *ApoI* and *BspTI* were useful in differentiation between mutton and chevon. *AluI* proved to be of value to discriminate between small and large ruminants. Application of this technique on adulterated meat samples of buffalo and cattle meat combinations could detect both animal species down to the level of 20% and 5% with *AluI* (specific for Cattle) and *HhaI* (specific for buffalo) respectively. It was possible to differentiate between cattle and buffalo meat samples using AluI enzyme alone.

KEYWORDS: PCR-RFLP, mt 12S rRNA gene, meat adulteration, cattle, buffalo

INTRODUCTION

Rapid urbanization and industrialization have led to growth of meat industry and its products. These changes also increase the chances of adulteration with meat of inferior and taboo species in meat and meat products. PCR-RFLP is one of the most popular techniques currently applied in biological research due to its easy application in routine surveys. Both nuclear and mitochondrial DNA (mtDNA) have been utilized for species identification using the PCR-RFLP method. mtDNA is commonly used because of its highly conserved sequences, maternal inheritance pattern, rapid evolutionary rate, and it is present in high copy numbers, making it easy to amplify (Romaino *et al.* 2014).

It was demonstrated that PCR techniques with universal primer pair ,in combination with PCR-RFLP allow identification of meat species occurring independently or in mixtures with other meat species. Keeping in view this fact the combination of this technology was used to identify the origin of specific meat among common meat animal species and the level of adulteration of cattle meat in buffalo meat.

MATERIALS AND METHODS

For standardization of technique, 4 commonly used meat species viz. cattle (8 samples), buffalo (10 samples), sheep (9 samples) and goat (10 samples) required for the present work were obtained from animals slaughtered at Deonar Abattoir, MCGM Mumbai. 17 coded meat samples from those sent to Animal Biotechnology Cell, Bombay Veterinary College, Mumbai, for species identification were also included in the study as samples of unknown origin . In order to evaluate the efficacy of technique in detecting species of origin from adulterated samples, the meat samples were mixed thoroughly in different proportions (each of two) as 50:50; 60:40; 70:30; 80:20; 90:10 and 95:5 of buffalo meat: Cattle meat (Beef). All meat samples were transported under ice-chilled condition (4 °C) and were stored at 20°C for further analysis. Total DNA from meat samples was extracted by Phenol Chloroform Iso-amyl alcohol method as described by Girish *et al.* (2011). The PCR for mitochondrial 12s rRNA was carried out using universal primers, forward primer (5-CAA ACT GGG ATT AGA TAC CCC ACT AT-3' and reverse primers (5'-GAG GGT GAC GGG CGG TGT GT-3')

described by Kocher *et al.* (1989). PCR products resulting from amplification of the12S rRNA gene were subjected to restriction enzyme digestion using *AluI, HhaI, BspTI* and *ApoI* (MBI Fermentas) in separate reactions. Digested products were analyzed on agarose gel.

RESULT AND DISCUSSION

In the present study mitochondrial 12S rRNA gene was used as molecular marker for identification of cattle, buffalo, sheep and goat. The four meat species could be differentiated by amplification of ~456 bp fragment of mt 12S rRNA gene using universal primer together with the restriction enzymes. DNA from all studied samples were extracted and amplified successfully. The universal primers used in PCR amplification generated fragments of approximately 456 bp. To validate the technique we initially carried out the experiment on meat samples of known origin and then on adulterated meat samples. All the PCR products were subjected to restriction enzyme digestion using four restriction enzymes in separate reactions. Analysis of restriction enzyme digests by agarose gel electrophoresis revealed that the RFLP profiles of each species tested were characteristic, further the number and sizes of restriction fragments generated were as anticipated.

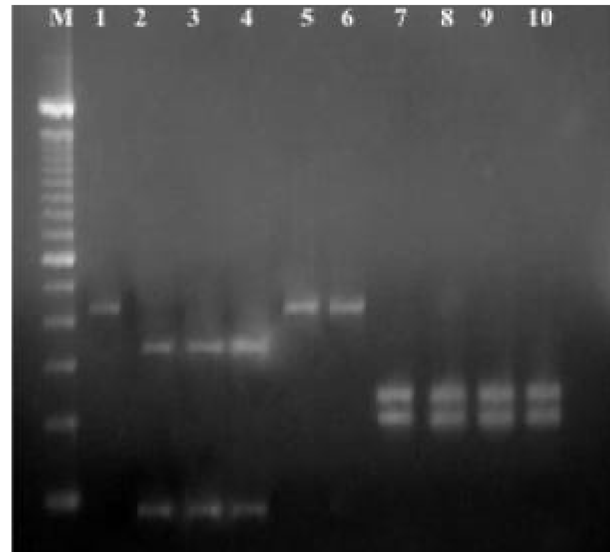

Fig. 1. PCR-RFLP Analysis: Alu I Digest
Lane M: 100bp DNA Ladder, 1-Negative Control, 2- Positive Control, 3-4: cattle, 5-6:buffalo, 7-8 : chevon, 9-10:mutton

With AluI the 456 bp PCR product obtained from buffalo remain undigested while in remaining three species the product was digested yielding two fragments; in cattle 359 + 97 bp and in sheep and goat showed similar pattern of size 246 + 210 bp (Figure 1). *HhaI* enzyme digested the amplicon from buffalo meat in two fragments of size 247 + 209 bp and there was no digestion of the PCR products from remaining species (Figure 2). *ApoI* could digest the PCR product of the sheep yielding in 329 + 127 bp, but there was no digestion in remaining species (Figure 3) (Mahajan et al. 2011). The amplicons from cattle, buffalo and sheep remained undigested with *BspTI* while that from goat was digested in 323 + 133 bp (Figure 4).

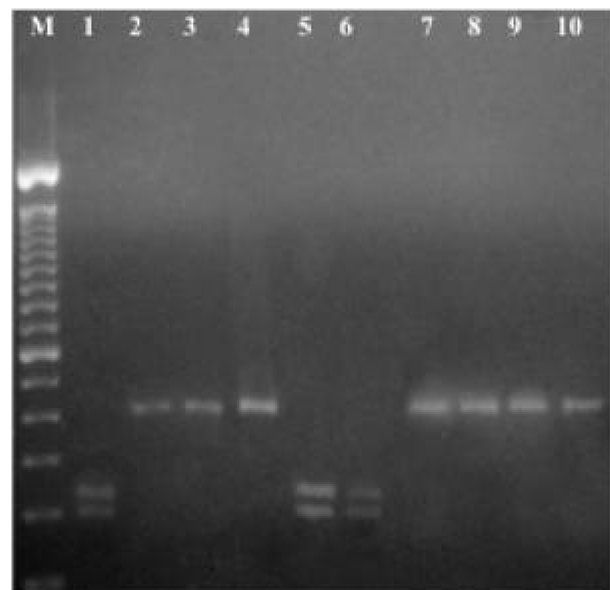

Fig. 2. PCR-RFLP Analysis: Hha I Digest
Lane M: M: 100bp DNA Ladder, 1-Positive Control, 2- Negative Control, 3-4: cattle, 5-6:buffalo, 7-8 :chevon, 9-10:mutton

It was possible to differentiate between cattle and buffalo meat samples using *AluI* enzyme alone (Figure 5). Further confirmation of species identity of cattle and buffalo was achieved with restriction enzyme *HhaI* which could digest the PCR product derived from buffalo into two fragments , no digests derived from cattle meat (Figure 6).

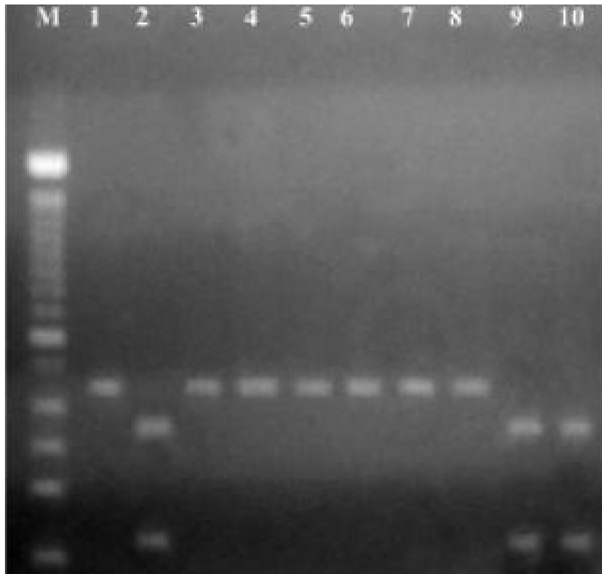

Fig. 3. PCR- RFLP Analysis: Apo I Digest
Lane M: 100bp DNA Ladder, 1-Negative Control, 2- Positive Control, 3-4: cattle, 5-6:buffalo, 7-8 :chevon, 9-10:mutton

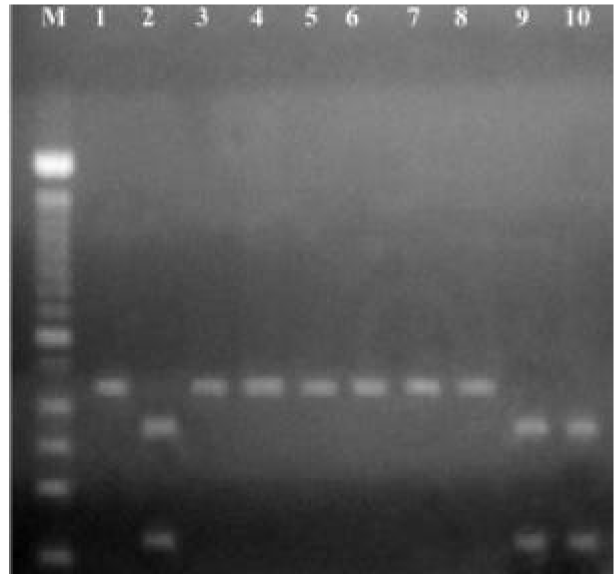

Fig. 4. PCR- RFLP Analysis: Bsp TI Digest
Lane M: 100bp DNA Ladder, 1-Positive Control, 2- Negative Control, 3-4: cattle, 5-6:buffalo, 7-8 : Chevon, 9-10:mutton

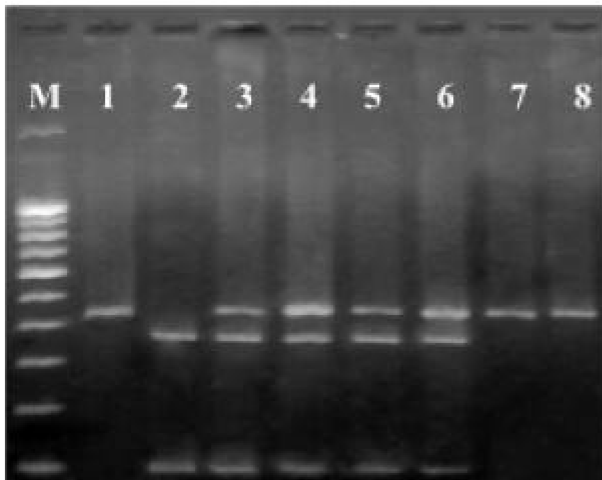

Fig. 5. PCR- RFLP Analysis: Alu I Digest
Lane M: 100bp DNA Ladder, 1-Negative Control, 2- Positive Control, 3-8: Sample A- F Buffalo: *Cattle (B:C)*.

A	B	C
B:C::50:50	B:C::60:40	B:C::70:30

D	E	F
B:C::80:20	B:C::90:10	B:C::95:5

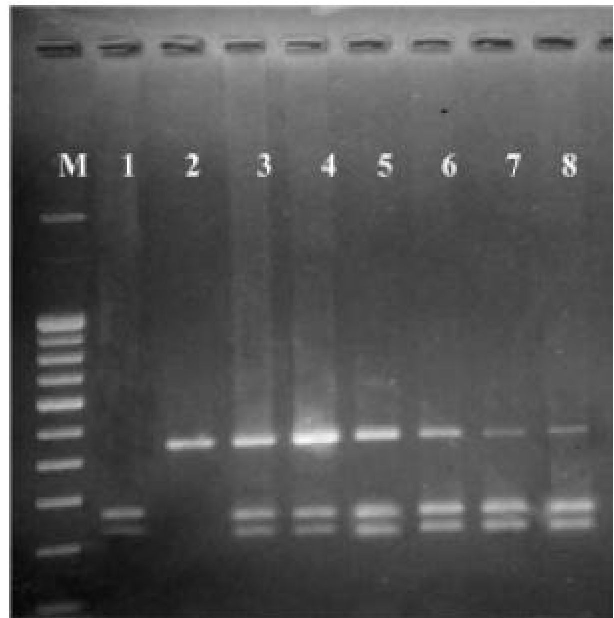

Fig. 6. PCR- RFLP Analysis: Hha I Digest
Lane M: 100bp DNA Ladder, 1-Positive Control, 2- Negative Control, 3-8: sample A- F Buffalo: Cattle (B:C).

A	B	C
B:C::50:50	B:C::60:40	B:C::70:30

D	E	F
B:C::80:20	B:C::90:10	B:C::95:5

AluI enzyme did not seem to be efficacious to differentiate sheep and goat since it could cut the amplicon generating fragments of similar sizes i.e. 246 and 210 bp in both species (Girish *et al.* 2005; Mahajan et al. 2011). However, this enzyme proved to be of value to differentiate large ruminants i.e. cattle and buffalo as well as it could discriminate between small and large ruminants.

ApoI and *BspTI* showed one restriction site each for the amplicons derived from sheep and goat respectively and thus were useful in differentiation between mutton and chevon. Both of these enzymes did not digest the PCR product derived from cattle and buffalo.

In order to confirm the validity of results, we also applied this technique for detection of meat animal species of unknown origin. It was possible to precisely identify the meat animal species in each of these samples using PCR-RFLP analysis of mt 12S rRNA gene results matched to the actual species in 100% cases.

Also an attempt was made for detection of animal species from deliberately adulterated meat samples with view to evaluate efficacy of this technique in its forensic applications. Buffalo and cattle meat mixed in different proportions was processed for this purpose by PCR-RFLP analysis of mt 12S rRNA gene. Precise identification of each species could be achieved in meat samples that were mixed at proportions of 50:50, 60:40, 70:30 and 80:20 with *AluI* (specific for Cattle) and in all proportions with *HhaI* (specific for buffalo). The species differentiation however could not be achieved with in *AluI* in proportions of 90:10 and 95: 5. It appears from the results that the PCR-RFLP analysis of mt 12S rRNA gene could be useful in detection of meat animal species from adulterated samples in situations where the proportion of meat from one animal species is at least 5% or more with HhaI and 20% or more *AluI*. The results were representative of 15 separate experiments with different samples. PCR-RFLP has been proven to identify the adulterated meat of Cattle and Buffalo species upto 20 % adulteration by *HhaI* and 5% by *AluI* restriction digestion.

In recent years, many investigators applied various typing methods including PCR amplification (Zarringhabaie *et al.* 2011) to identify species in meat. Velebit *et al.* (2009) established sensitivity of 0.5% of target species DNA; Kesmen et al. (2012) established the sensitivity to be 0.01% by conventional PCR assays and 0.0001% with real-time PCR assays. Whereas Zarringhabaie *et al.* (2011) able to trace each species meat when its portion in the mixture was less than 10%.

The PCR-RFLP analysis of mt 12S rRNA gene is the technique of potential value in precise identification of animal species like cattle, buffalo, sheep and goat. Only *AluI* was found to be sufficient to differentiate small ruminants (sheep and goat) and large ruminants (cattle and buffalo) and *ApoI* and *BspTI* was found to differentiate sheep and goat meat samples; *AluI* and *HhaI,* was able to differentiate between two combinations of meat (Buffalo and Cattle) down to the level of 20 and 5 %. But results did not correspond to the proportions of the mixtures. Application of the technique on meat samples of unknown origin further proved the efficacy of technique and reflected the prevalence of fraudulent practices in the meat industry. Future research in this area should focus on improvising the efficacy of this technique in detecting low level of adulteration and on developing assays for identification of other animal species.

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