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Rapid identification of goat (*Capra hircus*) and sheep (*Ovis aries*) species in raw meat using duplex PCR assay

N. Panwar, G. C. Gahlot*, Kritika Gahlot, M. Ashraf and Anoop Singh

Molecular Genetics Lab, College of Veterinary & Animal Science,
Rajasthan University of Veterinary & Animal Sciences, Bikaner 334 003, India

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ABSTRACT

Adulteration of high quality meat with their inferior/cheaper counterparts has become a common practice in the meat industry, which is not detectable by the naked eye or by eating. To circumvent this problem, molecular methods had been developed. The present study was carried out for detection of meat species with the use of cytochrome b gene variability by duplex PCR. Meat samples from goat, sheep and buffalo were utilized for molecular analysis. Genomic DNA was isolated from six samples of each species with some modifications. Mitochondrial cyotchrome b gene was amplified by conventional and duplex PCR using a common forward primer and species-specific reverse primer. PCR amplicons were resolved by agarose gel electrophoresis and for each species produce a characteristic band pattern in conventional and duplex PCR was obtained. The PCR products showed species-specific DNA fragments of 157 and 331 bps from goat and sheep respectively. The duplex PCR could detect upto 10 % of DNA of meat species. Thus, the duplex PCR was found to be a simple, reliable, sensitive and highly specific test for simultaneous detection of meat species.

Key words: Duplex PCR, Meat species, Mitochondrial Cyt b gene, PCR assay, Species-specific primer.

INTRODUCTION

Among all animal meats, goat meat (chevon) is the most preferred meat on the Indian subcontinent and despite being costly it is consumed by a large population. But, due to high cost of goat meat and to achieve monetary benefits, meat vendors often tend to adulterate goat meat with low priced meats such as sheep, buffalo, pig and some time the banned cow meat. For this reason, it is necessary to develop the process that would be fast, highly sensitive, cost effective and allow for high throughput. The current methods of discerning different animal species include the use of Short Tandem Repeat (STR) profiling, Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) techniques as well as performing analysis using separate species marker panels, real-time PCR and sequencing (Chikuni *et al.*, 1994; Fei *et al.*, 1996; Zhang *et al.* 1999; Matsunaga *et al.*, 1999; Spychaj *et al.*, 2009 and Deepak Kumar *et al.*, 2012). These methods, however, are often unreliable, time consuming and, in the case of sequencing, very expensive. Another problem often experienced with sequencing is that of mixed samples. If the sample is contaminated by another species' DNA, the results are unreliable as a result of the ability of universal species identification primers to bind many different species. This then requires an additional multiplex PCR to be performed.

On account of this, we need to develop and optimize the classic multiplex PCR, for the simultaneous and rapid detection of animal species, at least to identify the contamination in complex meat products. Multiplex PCR is a variant of PCR in which two or more DNA loci are simultaneously amplified in the same reaction. Since its first description in 1988, this method has been successfully applied in many areas of DNA testing (Henegariu *et al.*, 1997; Satish *et al.*, 2011). The use of multiple, unique primer sets within a single PCR mixture is to produce amplicon of varying sizes specific to different DNA sequences. By targeting multiple genes at once, additional information may be gained from a single test run that would otherwise require the use of reagents which require several times and a longer time to perform. Annealing temperatures for each of the primer sets must be optimized for them to work accurately within a single reaction and the amplicon sizes, that is, their base pair length, should be different to form distinct bands when visualized on gel electrophoresis.

A number of mt DNA genes are used as target for detecting or isolating different animal species in meat. Mitochondrial DNA owns several advantages over nuclear DNA. Mitochondrial DNA is presented in thousands of copies per cell and possesses many points of mutations allowing the

*Corresponding author's e-mail: gahlotgcbkn@rediffmail.com,

discrimination of even closely related species. Mitochondrial DNA is maternal inheritance and therefore is free of heterozygosity (Lockley and Bardsley, 2000). Cytochrome b (Cyt b) gene region is one of the conserved regions used as a molecular marker for this purpose (Hsieh *et al.*, 2001; Kumar *et al.*, 2011; Satish *et al.*, 2011 and Zarringhabaie *et al.*, 2011). Several NADH dehydrogenase genes (Lopez-Andreo *et al.*, 2005; Kesman *et al.*, 2009) and D-loop gene (Mane *et al.*, 2009; Kumar *et al.*, 2011 and Che Man *et al.*, 2012) are also used for species identification. In this study, the focus is on the determination of mt DNA (cytochrome-b) in unprocessed meat samples to optimize and develop a duplex PCR technique for detection of goat and sheep raw meat.

MATERIALS AND METHODS

Fresh goat and sheep meats were collected from a local market in Bikaner, Rajasthan (India). They were transported to the laboratory under refrigeration and were processed immediately or stored frozen at -20°C until used. Meat mixtures were prepared by adding 0.5%, 1%, 5%, 10% and 20% sheep meat in goat meat samples and vice versa. Genomic DNA was extracted from goat meat samples containing 0.5%, 1%, 5%, 10% and 20% sheep meat and from sheep meat samples containing 0.5%, 1%, 5%, 10% and 20% goat meat by conventional method as suggested by Ausubel *et al.* (1987) with certain modifications. The isolated DNAs were analyzed for quality using 0.8 % agarose gel electrophoresis and then stored at -20°C until used.

Primer and PCR amplification: Three primers were used, of which two were of goat and sheep species specific. The primers used were of the mammal specific primer set which consisted of the universal forward (SIM) and reverse species specific primers. The universal and species specific primer, which were published by Matsunaga et al. (1999), were used. The sequences of the forward and reverse primers were

Universal primer (forward): 5' -CCTCCCAGCTCCATC AAACATCTCATCTTGATGAAA-3'

Goat (reverse primer) : 5' CCTCCCAGCTCCATCAAACAT CTCATCTTGATG AAA-3'

Sheep (reverse primer) : 5' - CTATGAATGCTGTGGCTATT GTCGCA-3'

Simplex PCR: Polymerase chain reaction amplification was performed in a final volume of 25 μl, contained 2.50 μl of 5X PCR buffer, 1.0 μl $MgCl_2$ (25 mM),1.0 μl dNTPs, 1.0 μl Primer mix (10 pmole/μl), 1.0 ml Taq DNA polymerase (5 U/ml), 1.0 ml DNA template and 18 μl DNAse free water. Amplification was carried out at 94°C for 30 sec (denaturation), annealing at 60°C for 45 sec and extension at 72°C for 45 sec and repeated these steps for 35 cycles. The products of PCR amplification were electrophoresed on 0.8% w/v agarose gel containing 1% ethidium bromide solution @ 5 μl/100 ml at constant voltage of 80 V for 30 min in 1X TAE. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system.

To test the sensitivity of species specific primer, serial dilution of the DNA template ranging from 0.001ng to 5 ng were subjected to the reaction containing the individual species-specific primer. The specificity of PCR was analyzed by using 5 ng of each animal species from meat, and added to a mixture of species-specific primer set. Alternatively, the mixture of animal DNAs was subjected to an individual species-specific primer set.

Duplex PCR assay: For the simultaneous detection of each species, a duplex PCR was developed using each of the primer sets previously designed for simplex PCR. As for the simplex PCR, amplification was performed in a final volume of 25 μl contained 2.50 μl of 5X PCR buffer, 1.0 μl $MgCl_2$ (25 mM), 1.0 μl dNTPs, 1.5 μl Primer mix (50 pmole/μl) (0.5 μl common forward primer, 0.5 μl goat primer and 0.5 μl sheep primer), 1.0 ml Taq DNA polymerase (5 U/ml), 1.0 ml DNA template and 18 μl DNAse free water. Thermal cycling was programmed following the same procedure used for simplex PCR. The effectiveness of the duplex PCR system was determined by adding DNA mixture from each animal species in a mixture of species-specific primer set. The amplification was performed as described earlier. The sensitivity of the detection was analyzed using 0.8 % agarose gel by UV transilluminator.

RESULTS AND DISCUSSION

Mitochondrial DNA of cyt b gene fragments were amplified by conventional PCR from DNAs of meat samples using a common forward primer (SIM), goat specific reverse primer and sheep specific reverse primer as described by Matsunaga *et al.* (1999). Gradient PCR technique was employed to determine suitable annealing temperature (51–61° C) for amplification of cyt b gene of goat and sheep. An optimum annealing temperature of 57°C for goat and 58°C for sheep was found suitable for amplification. Amplicons of size 157 bp (Goat) and 331 bp (Sheep) were observed when amplified products were run on 0.8% agarose gel in gel electrophoresis apparatus and size of amplicons was confirmed by running parallel a 100 bp DNA marker (Fig.1). Meat of two closely related species, sheep and goat, were clearly differentiated, as fragments of these two species were widely different. The occurrence of band at 157 bp and 331 bp PCR product on gel electrophoresis confirms the presence

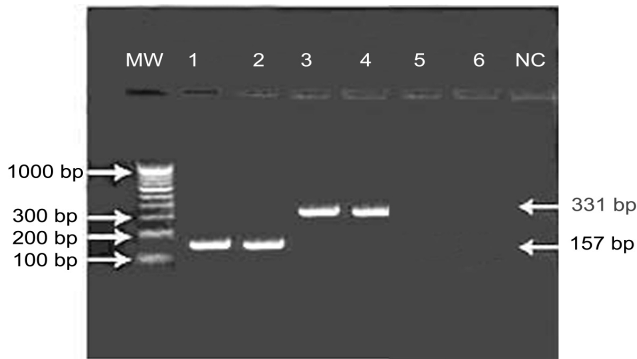

FIG 1: PCR amplification of mitochondrial cyt b region in goat and sheep: (Lane m) 100 bp DNA ladder; (lane 1and 2) goat (157bp amplicon); (lane 3 and 4) sheep (331bp amplicon); (lane 5 and 6) buffalo; (lane 7) negative control

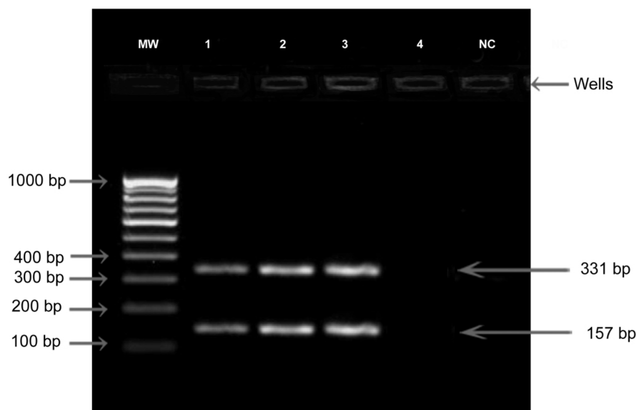

FIG 2: PCR amplification of mitochondrial cyt b region in different goat and sheep meat samples for validation of Duplex PCR: (lane MW) 100 bp DNA ladder; (lanes 1–3) different goat and sheep meat samples (157;331 bp amplicon); (lane 4) negative control.

of goat and sheep DNA, respectively (Fig.2) and does not require further analysis for confirmation.

Similar findings were reported by Kumar *et al.* (2011), who targeted mitochondrial D-loop gene for differentiation of meats from six food animal species including goat. Similar findings were reported by Satish *et al.* (2011), who targeted Cytochrome-b gene for differentiate the raw meats of Indian sheep, goat, cattle, pig and chicken. They differentiated meats of phylogenetically related species like sheep and goat from PCR products at DNA fragments of 157 and 331 bp respectively. The used technique could detect 20% adulteration of meat of one species into the meat of other species.

The possibility of cross amplification was precluded by analyzing goat specific primers with DNA extracted from sheep and buffalo meats. The 157 and 331 bp amplicon was evident in goat and sheep DNA, respectively and no amplification was observed in the DNA of other species including a negative control (Fig. 1). Repeatability of the PCR assay was confirmed 15 times by testing species-specific primers with DNA isolated from different goat and sheep meat samples, where invariably 157 and 331 bp amplicon was obtained (Fig. 2). The results revealed different specific amplified fragments of pure meat sources for goat, sheep and buffalo species. After mixing different portions of the mentioned meat sources, this method was able to trace less than 10% of the other species of meat in the mixture.

Results of this study support the findings of Meyer *et al.* (1994, 1995), Hopwood *et al.* (1999), and Partis *et al.* (2000) who reported that PCR could be used for identification of meat mixes at 1 and 0.5% levels.

Earlier, restriction digestion based PCR assays targeting different mitochondrial genes, namely cytochrome-b gene (Meyer *et al.*, 1995; Partis *et al.*, 2000; Sun and Lin, 2003; Maede, 2006, 12S rRNA gene (Girish *et al.*, 2005) and D-loop gene (Malisa *et al.*, 2006), have been employed for identification of goat meat. However, the goat specific assay developed in the present study, being a single step method, offers advantages over the previously reported techniques with respect to its simplicity, specificity and accuracy. The advantage of the present molecular methods is that there is no need to use restriction enzyme and restriction fragment length polymorphism (RFLP) methods to distinguish specific electrophoresis fragment on agarose gel. The occurrence of 157 bp PCR product on gel electrophoresis confirms the presence of goat DNA and does not require further analysis for confirmation.

To determine the detection limit of the PCR assay, amplifications were performed on two series of meat mixtures containing 0.5%, 1%, 5%, 10%, 20% and 100% (W/W) of each target species i.e. Goat and Sheep. Meat mixtures were prepared by adding 0.5%, 1%, 5%, 10% and 20% goat meat in different sheep meat samples and by adding 0.5%, 1%, 5%, 10% and 20% sheep meat in different goat meat samples. For each PCR series, it was observed that the lower the percentage of target meat in the admixture, the fainter the band obtained in the PCR with the corresponding primers. The detection limit (lowest meat percentage pro-ducing visible DNA amplification) of the assay was set on 1% for the particular species in meat mixtures. None of the primer pairs showed cross reaction with DNA of other species.

In recent years, many investigators applied various typing methods including PCR amplification to identify species in meat. For example, Meyer *et al.* (1994) detected 0.5% pork in beef using the duplex PCR technique. Their results revealed that PCR was the method of choice for

identifying meat species in muscle foods. They also detected 0.01% sow's protein in processed meat products using the nested-PCR technique. Partis *et al.* (2000) also detected 1% pork in beef meat. Hopwood *et al.* (1999) detected 1% chicken in lamb meat using PCR-RFLP.

The results of this study also showed that designed primers worked well in duplex manner and this method was sensitive and it was possible to trace each species meat when its portion in the mixture was less than 10%.

CONCLUSION

This study suggests an accurate analytical technique for goat and sheep meat identification, based on PCR analysis of the Cyt b gene of mitochondrial DNA. Developed technique is useful and feasible to trace meat adulteration and differentiate species present in mixed meat. Therefore, it can be suggested as a useful laboratory tool for species identification, especially for goat or sheep meat traceability in mixed meat.

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