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Research Article

Detection of Species-Specific Meat of Buffalo, Goat and Sheep by Multiplex-PCR Based Method

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Abstract: The production of buffalo's, goat's, and sheep's meat has considerable economic importance based on their high consumption in various industries. It is commonly found that undesirable mixing in meat may lead to allergy, religious, ethical or cultural objections of undeclared meat during processing. In the present study, the polymerase chain reaction (PCR) has been validated to identify three meats (buffalo, sheep and goat) as raw materials for products simultaneously in one mixture. A forward primer and reverse primers were designed on a conserved DNA sequence in the mitochondrial cyt b gene and species-specific DNA sequences for each species respectively. Selected samples of meats from buffalo, goat and sheep were applied for molecular analysis after being critically characterized. Genomic DNA was isolated from each species in triplicates. The PCR products showed species-specific amplicons of 155 bp, 274 bp and 331 bp for goat, buffalo and sheep respectively. The method based on genetic traceability using PCR might become useful for the consumers, food industry, and law enforcement and applicable to different raw meats and their products.

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INTRODUCTION

India is world's largest meat producer and standing eighth in rank in the world's meat production. In economic point of view Livestock sector of Indian agriculture industry, is one of the most important revenue generating sector and it is estimated as meat production is about 4.9 million tonnes. The availability of meat in India is only about 15gm/ person/day against the ICMR recommendation of 30gm/person/day [1]. Meat is rich in vitamins, minerals and proteins thus become an integral part of our diet. Meat provides omega 3 fatty acid and conjugated linoleic acid which are the source of many valuable nutrients and bioactive substances like taurine, carnitine etc [2-5].

Meat obtained from different animals have different nutritional and medicinal relevance which makes some of them, a commodity of high demand by industries as they can be used as a source of isolation and production of commercially important pharmaceutical products. Issues like health, social, religious and market completion play important role in people's ethical and economical concerns regarding meat [6, 7]. Buffalo, sheep and goat meats are cheap and widely available in Indian markets. Moreover, to get better profits, these easily available meats are commonly adulterated with other meat products. There is a need to develop rapid, accurate and specific methods for the identification of animal species specific meat which may protect our consumers from the defamatory practices of meat adulteration. To answer the challenges of above discussed reasons, various analytical approaches for example based on anatomical, histological, microscopic, organoleptic, chemical, electrophoretic, chromatographic or immunological, have been utilized for meat authentication [8-13]. Various analytical techniques like SDS-PAGE, ELISA and chromatography are not very effective for authentication of meat products because proteins in processed meat products are frequently degraded, denatured or damaged [14]. Consequently, DNA-based methods such as PCR are preferred to protein-based methods for species authentication.

Genetic traceability is the method which based on the identification of both animals and their products through the DNA analysis. The application of DNA based methods provides identification different levels, for example as individual traceability to insure food safety and traceability of individuals to their species to detect possible labelling adulteration [15]. When any DNA is amplified with the help of PCR, it provides very significant clue regarding species identification of its source, thus many experiments have been based on this principle making it an alternative method for the determination of the existence of fraud or other species contamination in any product [16, 17]. A universal DNA marker or specific marker which is only found in a particular species of animal is used in the method to mark the authenticity of the method.

The DNA markers mainly come from the

mitochondrial genome, including the cytochrome b gene [18], the 16S rRNA gene [19], the 12S rRNA gene. Various DNA genes of mitochondria may be used as the target for detection and identification meats from animal species. It is observed that Mitochondrial DNA might be advantageous over nuclear DNA when using it as a source of species specificity as it has not changed a lot during the course of evolution and contains several conserved genes. Mt DNA is present in thousands of copies per cell. It allows the discrimination of even closely related species and mt DNA follows maternal inheritance and therefore it is free of heterozygosity [20] Cyt b gene is one of the genes which has conserved regions and it has been used as marker for the present study [21, 22]. This study focuses on the isolation of mitochondrial DNA from raw meat sample and using it as template DNA for PCR. The multiplex PCR has been developed and optimized for detection of buffalo, goat and sheep unprocessed meat.

MATERIALS AND METHODS

Sample collection and preservation

Fresh and raw meat samples of buffalo, goat and sheep were collected from different local meat shops, in separate clean, cold and sterilized glass containers. Samples were kept on ice and transported to the laboratory to be stored at -20^{0}C until used for analysis.

DNA Extraction

Fresh meat samples from three different genera of animals (buffalo, goat and sheep) were collected from local meat shops of Lucknow and Barabanki (U.P.), India. The collected samples were transported to the laboratory under refrigerated conditions and stored at -20^{0}C till further use for analysis. DNA was extracted from the samples using BioEra Mt DNA isolation kit according to manufacturer's instructions (BTK 120958, BioEra Life Sciences, Pune India). Subsequently, the quality of DNA was checked on 1% agarose gel (Fig. 4).

Oligonucleotide primers

Highly purified Oligonucleotide primers used in this study were purchased from *Micelles Life Sciences pvt. Ltd*. Primer designing was done as described by Matsunaga *et al*., 1999 [8]. The sequences of all oligonucleotide primers used in the study are tabulated as table 1. The primers were diluted in equal proportion with double distilled water and stored at -20 ^{0}C for experimental work.

Table 1: Sequences of primers used for Multiplex PCR of *cyt b* gene

S.N.	Name	Primer	Sequences (5'- 3' direction)	No. of Bases
1	CFP*	F	GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA	38
2	GOAT	R	CTCGACAAATGTGAGTTACAGAGGGA	26
3	BUFFALO	R	CTAGAAAAGTGTAAGACCCGTAATATAAG	29
4	SHEEP	R	CTATGAATGCTGTGGCTATTGTCGCA	26

*(Common Forward Primer)

Primer validation by *in-silico* method

Species specific primers were validated by SnapGene tool (http://www.snapgene.com) using all three species specific cytochrome b gene sequences retrieved from NCBI. For the validation of the primers used in this study, the sequences of cytochrome b gene from goat (Accession no.: KY348330), buffalo (Accession no.: MG820631) and sheep's (Accession no.: KU681224) sequences were retrieved from NCBI. The primers of specific size and sequence are used against all three species and after experiments it was observed that they are found significant. The results of *in-silico* primer validation are given in the Fig. 1, Fig. 2 and Fig. 3 for buffalo, goat and sheep respectively. It was observed that primers were competent enough to amplify respective sequences of the particular species.

PCR amplification of the gene

The primers were used to amplify highly polymorphic regions within buffalo, goat and sheep mitochondrial cytochrome *b* gene (cyt*b*). The physical parameters like melting temperature, self-complementarities and secondary structure were checked using online tool *OligoCalc (http://www.basic northwestern.edu/ bio-tools/ OligoCalc.html)* and the cross-reactive nature of the primers were also observed by another online tool AutoDimer (http:// www.cstl.nist.gov/ strbase/AutoDimerHomepage/ AutoDimerProgramHomepage.htm). For conventional PCR, 0.5µl CFP (common forward primer) and 0.5µl of reverse primer for each species were used. CFP was used as forward primer for all three species (Goat, Buffalo and Sheep). Having confirmed species specificity of each reverse primer independently, a multiplex PCR was done by using Qiagen multiplex PCR kit (Cat No./ID: 206143) by mixing all primers in single reaction but targeting DNA of single species. Initially, all the primers were mixed in equal proportion and 4 µl of this cocktail was incorporated in the PCR. The PCR reactions were optimized with respect to primer concentrations. Primers (table 1) were mixed in the ratio of 1:0.25:4:0.6 for CFP: G: B: S (Common Forward Primer: Goat: Buffalo: Sheep) and were used together for the multiplex PCR. The PCR protocol used for amplification of mitochondrial cyt*b* comprised of an initial denaturation step at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 30 seconds and a final extension step at 72°C for 10 minutes.

The interpretation of results visualization and amplification

PCR products were resolved by 1% agarose gel electrophoresis and each species produced a characteristic band pattern at constant voltage 90 V for 60 minutes. The amplified products were visualized as a single compact band of expected size under UV light and documented by gel documentation system.

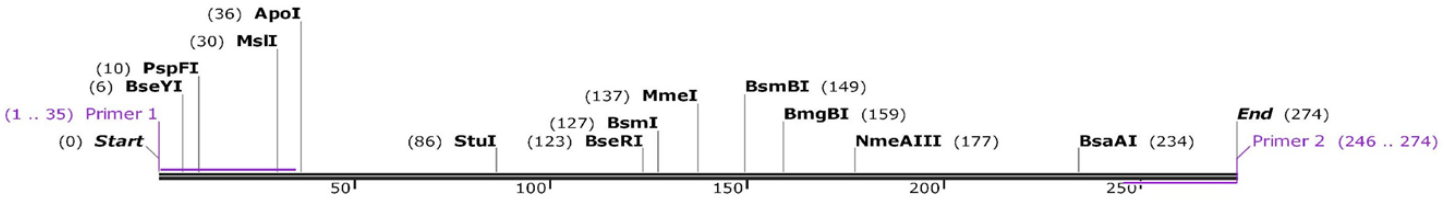

Fig. 1: *In-silico* Primer validation of buffalo's species-specific cytochrome b gene

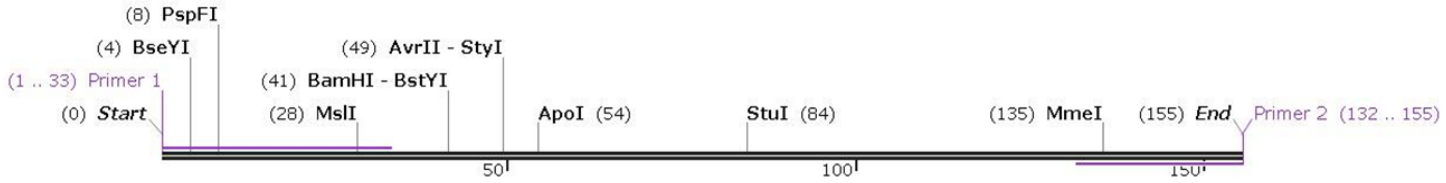

Fig. 2: *In-silico* Primer validation of goat's species-specific cytochrome b gene

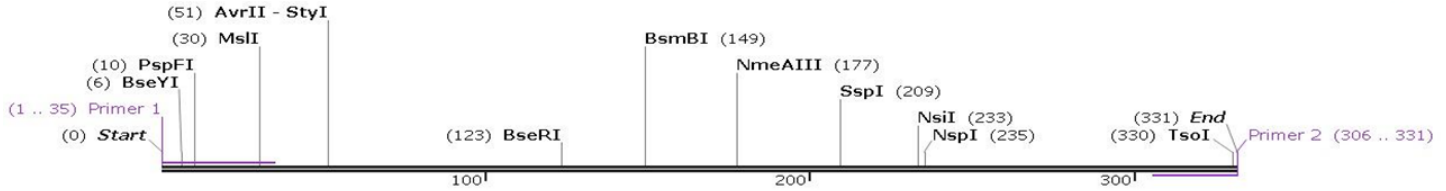

Fig. 3: *In-silico* Primer validation of sheep's species-specific cytochrome b gene

RESULTS AND DISCUSSION

Meat adulteration may be detected by molecular techniques and PCR based techniques that have proved their mettle in identifying meats of domesticated animals, meat products and various fish varieties [23-25]. The above studies used mitochondrial cytochrome b for amplification and the PCR products were studied by RFLP. The method based on multiplex PCR technique was able to identify quality of meat of some domestic animals [8]. Jain et al., 2007 also used cytochrome b gene variability was also become helpful in the detection and identification of meat species using multiplex PCR as a tool [26]. The presence of species-specific DNA sequences and detecting such sequences has opened a new era of molecular genetics for identification of respective species using the assays based on the DNA hybridization probes, PCR or RFLP.

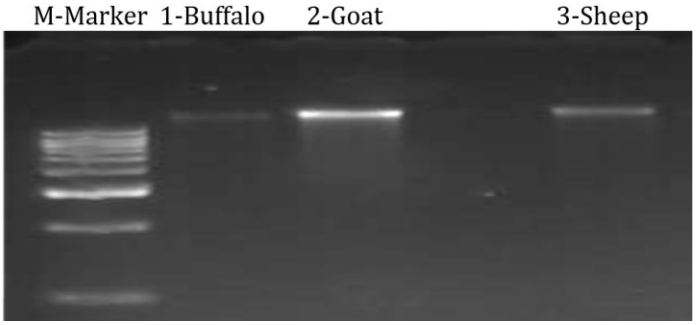

Fig. 4: Agarose gel Electrophoresis of genomic DNA isolated from meat samples (M-Marker: 1kb)

The primers in the multiplex PCR amplified target sequences at the efficiency comparable to conventional PCR. Amplified PCR products from three species ranged from 155 to 331 bp. By using this approach, we could discriminate between buffalo's, goat's and sheep's meat samples.

The three pairs of primers amplified the expected fragment of 274bp for buffalo, 331bp for sheep and 155bp for goat (Figure 5). The difference in size of amplicon was due to the presence of highly polymorphic regions within buffalo, sheep and goat mitochondrial cyt b gene. The successful amplification of these polymorphic regions in cyt b gene from the target species produced a characteristic band pattern for each species.

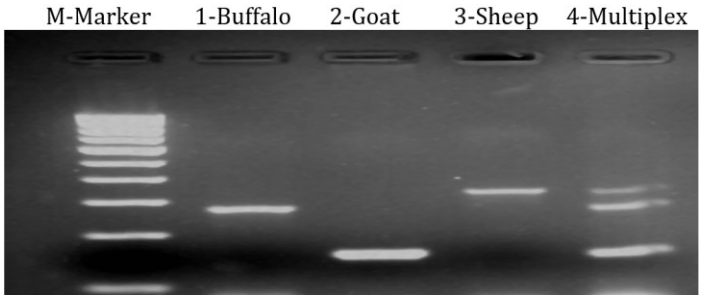

Fig. 5: Agarose gel electrophoresis of PCR products amplified with Multiplex-PCR for the fragments of Buffalo, Goat and Sheep Cyt b gene (Lane 1 represents product from Buffalo (274bp), Lane 2 represents product from Goat (155bp), lane3 represents product from Sheep (331bp), lane 4 represents the Multiplex PCR products of Buffalo, Goat and Sheep and M represents 100bp DNA ladder).

Common adulterations of meat products are the substitution of higher value meat by non-declared meat or the omission of a declared meat species. The PCR amplification methods for various species-based DNA sequences of mitochondrial genome is most reliable and widely used for the species identifications. There are many clues in previous reports on the counterfeiting of goat's meat.

CONCLUSION

In recent studies, DNA from tissues of meat was amplified by the PCR assay for identification and discrimination of species of origin in meat. In light of the knowledge of molecular methods for the identification of species, the aim of this study was to identify as well as discriminate species-specific meat (buffalo, goat and sheep) by developing multiplex PCR assay. In the study, PCR product amplification approaches using mitochondrial cyt b and its variability were applied. The proposed multiplex PCR is a reliable, sensitive and fast method that could be used on a routine basis. It is possible to detect via a single step process, buffalo's, goat's and sheep's meat with a good sensitivity. This method could revolutionize detection of adulteration in meat and could prove to be very helpful for meat and pharmaceutical industries and also for government regulation authorities to keep a proper quality control check on meat products.

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CONFLICT OF INTEREST

There is no conflict of interests between the authors.

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