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Research Article

Meat Adulteration in Cooked Mutton Kebab with Cattle and Buffalo Meat and its Detection Using Mitochondrial DNA (mtDNA) Based Multiplex PCR

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Abstract

Background and Objective: The incorrect labeling of meat products may have remarkable negative consequences on meat industry. The adulteration of meat needs to be taken care of in order to save the victimization of consumers. Keeping in view the adulteration chances in indigenous meat products of Kashmiri cuisine, the present study was carried out to study the detection of cattle and buffalo meat in cooked mutton kebab by mitochondrial DNA (mtDNA) based multiplex Polymerase Chain Reaction (PCR) method under laboratory conditions. **Materials and Methods:** Kebab was prepared as per the standardized processing schedule using pure mutton, cattle and buffalo meat and their admixtures in the ratios of 60:20:20, 80:10:10, 90:05:05 and 98:01:01, respectively. The trials were carried out in triplicate. The mtDNA from test samples was extracted as per the standard protocol. Various primers were used in the study for carrying out the PCR analysis. **Results:** The *cyt b* gene fragments of sizes 124, 472 and 585 bp for buffalo, cattle and sheep, respectively were amplified by the primers used in the study. In the mixed cooked kebab the detection of cattle and buffalo species was possible to a level of 1%. The characteristic band pattern for each species was achieved through successful amplification of *cyt b* gene fragments of mtDNA of the target species with band intensities of cattle and buffalo decreasing corresponding to their reduction level from 20-1%. **Conclusion:** In conclusion, the processing cooking (moist heating) and addition of non-meat ingredients showed no hindrance in the detection of meat species. Thus, the multiplex PCR procedure used in the present study proved to be effective and reliable in detecting the adulteration of cooked mutton kebab with cattle and buffalo meat up to 1% level.

Key words: *Cyt b* gene, kebab, meat adulteration, multiplex PCR, mtDNA

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In 13th century A.D., the first case of fraudulent substitution was reported by Thornton[1] at Florence in Italy. The practice of fraudulently adulterating the high priced meat with cheap one is a serious problem throughout the world. Therefore, with regard to economic, health, religious and ethical issues, detection of less desirable or objectionable species in meat products is of utmost important besides ensuring compliance with legislation and fair trade[2]. In the recent past, enzyme-linked immunosorbent assays[3] and protein profiles[4] have been utilized for identification of meat species adulteration. Of late, new methods like molecular authentication based on PCR amplification have been used for species authentication in meat products with great success[5-8]. As per Cheng *et al*.[9], the DNA-based analysis has many advantages like DNA ubiquity from all cell type of an individual contains identical genetic information that is independent of the origin of samples. Additionally, there is abundant information in DNA as compared to proteins due to degeneracy of the genetic codes. Moreover, due to the stability of DNA molecule, its extraction and analysis from various different samples is practicable. Analysis of species-specific mitochondrial DNA sequences through PCR has been the most common method used for meat species identification in food[10-14] and animal feedstuffs[15-17]. Presently, for identification of various species in the meat and meat products, PCR assays have been employed that target genomic and mitochondrial DNA[18-21]. Species specific primer pairs have also been employed for certification of poultry and mammalian species[19-22]. For fast and authentic recognition of chicken, cattle and buffalo meat, species-specific PCR assay was developed with efficacy even for the samples that are blended heat processed meat products containing the non-targeted species[23].

The present study was thus carried out to study the species-specific detection of cattle and buffalo meat adulteration in cooked raw mutton kebab emulsion using multiplex PCR of *cyt b* gene fragments under laboratory conditions.

MATERIALS AND METHODS

Hot boned mutton, cattle and buffalo meat from leg portion of the respective dressed carcass as well as the respective visceral fat were procured and packaged in properly labeled low density polythene (LDPE) bags.

Kebab processing and sampling: The cooked form of product of mutton, cattle and buffalo meat were prepared according to the standardized processing schedule and recipe of Salahuddin[24] with slight modifications. In accordance with the formulation, weighed portions of minced meat and fat from each species were taken for the desired meat component for the treatments (admixtures) after thorough mixing (Table 1). Random samples of cooked kebab of about 50 g each were drawn separately from the four respective types of kebab. The samples so obtained were packaged in properly labeled LDPE bags and frozen stored at -20°C.

Mitochondrial DNA extraction: The mtDNA from test samples was extracted as per the standard protocol described by Wilson[25] with some modifications. About 300 mg aliquot of the frozen test sample was cut with a sterile scalpel, transferred to autoclaved porcelain mortar and ground thoroughly by pestle with additions of liquid nitrogen. The homogenate was transferred into a sterile 15 mL tube and liquid nitrogen was allowed to evaporate. Lysis buffer-ST (0.5 mL) was added to the tube along with 5 µL proteinase K and 10% SDS (100 µL) to make final concentration of the latter to 2%. The homogenate was incubated for 12-16 h (overnight) at 55°C; the lysate was transferred to an autoclaved 15 mL centrifuge tube and equal volume 0.5 mL of tris saturated phenol (pH 8.0) was added and mixed gently for 10 min. The lysate was then centrifuged at 10,000 rpm and 15°C for 10 min and supernatant transferred into a 2 mL centrifuge tube and half the volume of tris saturated phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed gently for 10 min. It was centrifuged at 10.000 rpm and 15°C for 10 min and supernatant transferred into 2 mL centrifuge tube and equal volume of chloroform: isoamylalcohol (24:1) was added and mixed gently for 10 min and centrifuged at 10,000 rpm and 15°C for 10 min. The supernatant was collected into a 2 mL centrifuge tube followed by addition of 1/10th volume of 3 M sodium acetate (pH 5.5) and equal volumes of isopropyl alcohol. The tubes were slowly swirled to precipitate the DNA which was then washed thrice with 70% ethanol, air dried and then dissolved in 200 µL volume autoclaved triple distilled water using properly marked 2 mL tubes. The DNA samples (stock solution) were stored at -20°C until further use. Quality, purity and concentration of the extracted DNAs were checked by agarose gel electrophoresis and spectrophotometry.

Primers for PCR: Various primers used in the present study were as per Zarringhabaie *et al*.[26] and are listed in Table 2. The

Table 1: Proportions of meat and fat used in the formulation for kebab

	Treatments			
Percent ingredients	T_1	T_2	T_3	T_4
Mutton	48 (60)	64 (80)	72 (90)	78.40 (98)
Cattle meat	16 (20)	8 (10)	4 (5.0)	0.80 (1.0)
Buffalo meat	16 (20)	8 (10)	4 (5.0)	0.80 (1.0)
Mutton fat	12 (60)	16 (80)	18 (90)	19.60 (98)
Cattle meat fat	4 (20)	2 (10)	1 (5.0)	0.20 (1.0)
Buffalo meat fat	4 (20)	2 (10)	1 (5.0)	0.20 (1.0)
Total	100	100	100	100

T_1, T_2, T_3 and T_4 indicate mixed meat kebab with mutton, cattle and buffalo meat as 60:20:20, 80:10:10, 90:05:05 and 98:01:01, respectively

Table 2: Primers used in multiplex PCR

Name	Primer type	Sequences (5'-3')	Size (bp)
Common	Reverse	TGTCCTCCAATTCATGTGAGTGT	-
Buffalo	Forward	TCCTCATTCTCATGCCCCTG	124
Cattle	Forward	TCCTTCCATTTATCATCATAGCAA	472
Sheep	Forward	TACCAACCTCCTTTCAGCAATT	585

primers were procured from M/s RFCL Ltd., New Delhi and were supplied in freeze-dried form and were stored at -20°C until their usage.

PCR analysis: The multiplex PCR was carried out in a thermocycler using 0.2 mL thin wall PCR tubes. Twenty five microliters PCR reaction volume comprised of 1.6 µL $MgCl_2$ (1.5 mM), 0.5 µL M dNTP (0.2 mM), 4.5 µL of common reverse primer and 1 µL of each forward primer of sheep, cattle and buffalo (0.015 mM), 2 µL of template DNA (50 ng), 2.5 µL of 10X PCR buffer, 0.06 µL Taq DNA polymerase (0.3 U) and remaining volumes (10.84 µL) of autoclaved triple distilled water. The optimized PCR protocol comprised of initial denaturation for 3 min at 94°C, followed by 34 cycles of denaturation for 30 sec at 94°C, annealing at 60°C for 45 sec, extension at 72°C for 45 sec and a final extension at 72°C for 10 min. The PCR products were electrophoresed at 85 V for 2 h in 2% agarose gels after the wells were charged with 5 µL of DNA preparations mixed with 1 µL of 6X gel loading buffer dye and viewed under UV trans-illuminator gel documentation after staining with ethidium bromide. The sizes of PCR products were determined in relation to a 100 bp DNA ladder.

RESULTS AND DISCUSSION

The results of multiplex PCR profile of cooked Kebab has been shown in Fig. 1. The expected species-specific band patterns were exhibited by amplified fragments of multiplex PCR. The amplified bands of *cyt b* gene fragments for buffalo, cattle and sheep were of the size of 124, 472 and 585 bp, respectively. Three bands representing the meats of three species (sheep, cattle and buffalo) were found for each mixed meat cooked kebab sample, while as in case of pure mutton, cattle and buffalo meat kebab, only one band of the respective species was observed. In the mixed cooked kebab the detection of cattle and buffalo species was possible to a level of 1%. Further, in case of pure meat samples, band intensities were of similar intensity. The characteristic band pattern for each species was achieved through successful amplification of *cyt b* gene fragments of mtDNA of the target species with band intensities of cattle and buffalo decreasing corresponding to their reduction level from 20-1%. Due to DNA degradation during cooking, the overall band intensities for sheep, cattle and buffalo *cyt b* gene fragments were lower. In the present study, the multiplex PCRs for cooked kebab were repeated thrice with reproducibility of 100%. Abd El-Nasser *et al.*[27] reported that meat species adulteration conflicting food labeling laws is a problem throughout world and has raised concerns of food safety. Thus, there is need to detect meat species by quick and reliable methods for the quality control and to ensure food safety[28]. The speciation becomes difficult in cooked samples as the cooking causes extensive changes in the meat tissue during heat treatment[29]. However, Ioja-Boldura *et al.*[30] reported that the short nucleic acid sequences survive cooking processes and could be used for the purpose of meat speciation. Analysis using nucleic acid based technique has been widely used in various fields, thus popularized for feed or food adulterant identification and differentiation[15].

The DNA based PCR assays are widely being used for identification and differentiation of species origin of meat and meat products in the recent past[6-8,31-33]. The advantages being high heat stability and highly conserved nature of DNA[18] and improved probability of positive results even in highly fragmented DNA of extremely processed meat products[34]. Ballin[35] also has reported that the unique variability and diversity of genetic code allows the discrimination of even closely-related species. Detection method based on mtDNA can further improve the sensitivity because of the fact that as against just a few sets of genomic DNA, there are thousands of copies of mtDNA per cell and are easier to be extracted[9]. As per Singh and Neelam[36] and Unajak *et al.*[28] 12, 16 and 18S

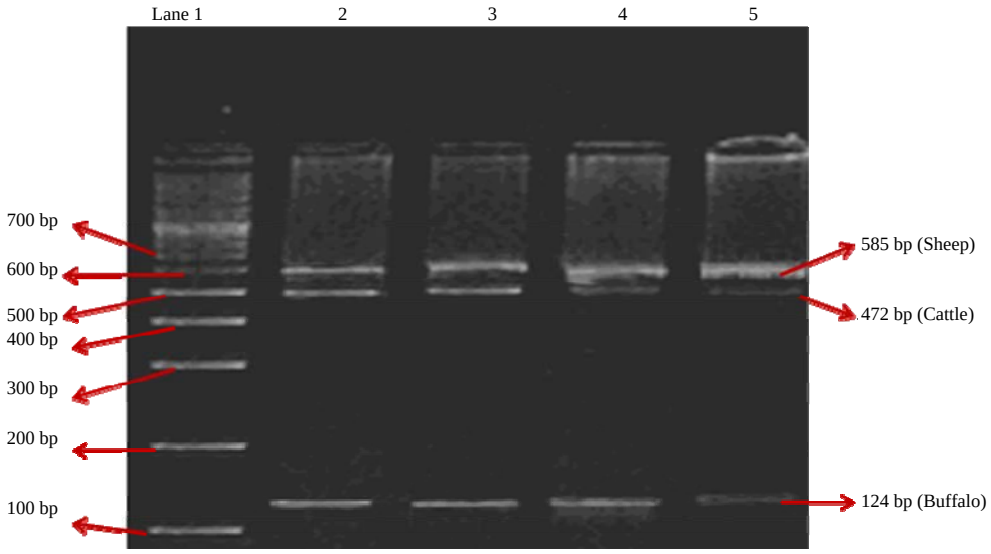

Fig. 1: Species-specific multiplex PCR Profile of *cyt b* gene fragments of sheep, cattle and buffalo from cooked kebab, Lane 1: DNA ladder of 100 bp, Lane 2-5: Mixed meat (mutton:cattle:buffalo meat in the ratio of 60:20:20, 80:10:10, 90:05:05 and 98:01:01, respectively)

rRNA, actin, cytochrome b (*cyt b*), cytochrome oxidase-II, NADH dehydrogenase 5/6 and mtD-loop are the genes to be targeted for amplification. Amongst these, the variation of mitochondrial *cyt b* gene has been a rich source of phylogenetic inference in a wide range of animal species. It has been reported that under different meat processing conditions there are very less chances of mtDNA degradation, thereby making it ideal for meat species identification in processed samples[13]. Further, a higher copy number of mtDNA ensures the availability of sufficiently large quantity of PCR product even in samples which have undergone intense fragmentation of DNA[37]. In the present study, the lowest detection level was up to 1%. Detection of meat speciation at similar levels of addition in mixed meats has also been reported by Rodriguez *et al.*[38]. Further as there was a progressive decrease in the level of cattle and buffalo meat from 20-1% in the mixed meat cooked kebab, the mtDNA concentration of cattle and buffalo meat (in the extracted mtDNA) also showed progressive decrease. Subsequently, the band intensities of cattle and buffalo meat amplified DNA also showed progressive decrease because of the fact that the band intensities and quality of mtDNA are correlated. These results on cooked kebab were in accordance with the findings of others[11,39,40]. Moreover, in the present study, due to DNA fragmentation during cooking, the band intensities of *cyt b* gene fragments of cattle and buffalo meat in cooked kebab were lower, thereby confirming the reports of other researchers[41,42] that heat treatment affects the quality of DNA.

CONCLUSION

The processing cooking (moist heating) and addition of non-meat ingredients showed no hindrance in the detection of meat species. Thus, the multiplex PCR procedure used in the present study proved to be effective and reliable in detecting the adulteration of cooked mutton kebab with cattle and buffalo meat up to 1% level.

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