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Detection of Adulteration in Uncooked Kashmiri Mutton Product (Rista) with Cattle and Buffalo Meat

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Abstract

The Jammu and Kashmir is a state in India where people are predominantly non-vegetarian, more so in Kashmir valley where meat and meat products are consumed since ages as exemplified by the world famous Kashmiri cuisine, *Wazwan*, with *Rista* being one of the predominant dishes. The fraudulent adulteration of superior quality meat with inferior quality meat is a practice that has been observed all over the world. The meat industry in India is largely unorganized; hence adulteration or substitution of meat in meat products is likely to be practiced. In view of such likely problem in indigenous meat products of Kashmiri cuisine (*Wazwan*), the present work was carried out to study the detection of cattle and buffalo meat in uncooked mutton *Rista* by mitochondrial DNA (mtDNA) based Multiplex polymerase chain reaction (PCR) method under laboratory conditions. The *Rista* emulsions were prepared from mutton, cattle and buffalo meat in the ratios of 60:20:20, 80:10:10, 90:05:05 and 98:01:01 respectively. The primers used in the Multiplex PCR produced characteristic band pattern for each species by amplifying *cyt b* gene fragments of mtDNA of the target species. The size of the amplified bands of *cyt b* gene fragments was 585, 472 and 124bp for mutton, cattle and buffalo meat respectively. Further, the band intensities progressively decreased for cattle and buffalo meat from mixed meat products with their corresponding decreasing level from 20 to 1%. It is thus concluded that the Multiplex PCR is highly effective in detecting adulteration of uncooked mutton product with cattle and buffalo meat up to 1% level.

Keywords: Meat adulteration, multiplex PCR, mutton, rista

Introduction

The higher demand for meat and meat products accompanied by their escalating cost makes them prone to fraudulent adulteration, substitution and mislabeling. The determination of food authenticity and the detection of adulteration are major issues in the meat industry and are attracting increasing amount of attention. Even from religious and ethical reasons, detection of adulteration is of utmost importance (1). Enzyme-linked immunosorbent assays (2) and protein profiles (3) have also been used for meat species identification. Assays based on polymerase chain reaction (PCR) are the method of choice for identification of meat species (4). Various researchers have reported that species-specific PCR analysis of mitochondrial DNA sequences is commonly used method for

identification of meat species in animal feedstuffs (5-7) and food (8-13). The PCR based assays targeting genomic and mitochondrial DNA are being used for the identification of meat species (14-17). The Using species-specific primer pairs for detection of species origin of meat have also been employed for certification of mammalian and poultry species (15-19).

In the state of Jammu and Kashmir, the people are predominantly non-vegetarian, more so in Kashmir valley where meat and meat products are consumed since ages as exemplified by the world famous Kashmiri cuisine, *Wazwan*, with *Rista* being one of the predominant dishes. The fraudulent adulteration of superior quality meat with inferior quality meat is a practice that has been observed all over the world (20).

In view of such likely problem in indigenous meat products of Kashmiri cuisine (*Wazwan*), the present work was carried out to study the detection of cattle and buffalo meat in uncooked mutton *Rista* by mitochondrial DNA (mtDNA) based Multiplex polymerase chain reaction (PCR) method under laboratory conditions.

Materials and Methods

Material and Preparation of Rista: Meat (mutton, cattle and buffalo meat) from leg portion of the respective dressed carcass as well as the respective visceral fat were procured from local market. The product was prepared according to the standardized processing schedule and recipe of Samoon (21) with slight modifications. The basic formulation for *Rista*, prepared under various controls, viz. mutton (CM), cattle (CB) and buffalo (CC) as well as treatments, viz. admixture of three meats respectively in percentages of 60:20:20 (T_1), 80:10:10 (T_2), 90:5:5 (T_3) and 98:1:1 (T_4) is presented in Table 1. In total three experimental trials were conducted under this study.

Preparation of Uncooked Rista Emulsion: The deboned lean meat from each species (sheep, cattle and buffalo) separately was cut into chunks of size 2-2.5 square inch size. The meat in chunks of each species so obtained was subjected to comminution by traditional manual pounding with a wooden hammer called on a flat and smooth round stone called "*Maz-Kaene*" (*Maz* = Meat, *Kaene* = Stone) until it attained a course ground state. All visible connective tissue, tendons, ligaments and blood vessels were separated manually during mincing. Pounding was continued until a fine pasty consistency of minced meat was obtained. In accordance with the formulation (Table 1), weighed portions of minced meat from each species were taken for the desired meat component for control and treatments (admixtures) after thorough mixing. Fresh fat from each species was also finely minced separately and portioned in a similar manner to that of the minced meat. The minced meat and fat component of each control/ treatment was then mixed together and pounded during which common salt and large cardamom seeds were added with periodical sprinkling of required amount of chilled water. Pounding was continued until proper dispersion of lean and fat was obtained and the resultant emulsion exhibited a characteristic cohesiveness, binding and fluidy consistency, traditionally called as "*Macchwor*".

Sampling of Uncooked Products: Random samples of about 50g each were taken from the uncooked emulsion for *Rista* under all the three pure meat controls and their four admixture treatments. The samples so obtained were packaged in properly labeled LDPE bags and frozen stored at -20 °C. For DNA extraction, aliquots were taken from these samples.

Extraction of Mitochondrial DNA: The chemicals utilized for mtDNA extraction from the test samples were Lysis buffer, *Proteinase-K,* TE Buffer, Phenol (Tris saturated, pH 8.0), 10% Sodium DodecyleSulphate (SDS), Chloroform, Isoamyl alcohol, Isopropyl alcohol, Ethanol, 3M Sodium acetate (pH 5.5). The mtDNA from test samples was extracted as per the standard protocol (22) with some modifications. About 300 mg aliquot of the frozen test sample was cut into small pieces with a sterile scalpel and transferred

to autoclaved porcelain mortar. The sample pieces were ground thoroughly by pestle with additions of liquid nitrogen. The test sample homogenate was transferred into a sterile 15 ml tube and liquid nitrogen was allowed to evaporate. Lysis buffer - ST (0.5ml) was added to the tube along with 5ìl *Proteinase K* and 10% SDS (100ìl) to make final concentration of the latter to 2%. The homogenate was incubated for 12-16 hours (overnight) at 55°C. At the end of incubation, 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. The supernatant was 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. The supernatant was 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 2ml centrifuge tube followed by the addition of 1/10th volume of 3M 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 and air dried and then dissolved in 200 ìl 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 used for PCR: Various primers used in the present study were as per (23) and are listed in Table 2. The 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.

Multiplex PCR Analysis: The Multiplex PCR was carried out in a thermocycler using 0.2 ml thin wall PCR tubes. The 25ìl PCR reaction volume comprised of 1.6 ìl $MgCl_2$ (1.5 mM), 0.5 ìl M dNTP (0.2 mM), 4.5 ìl of common reverse primer and 1 µl of each forward primer of sheep, cattle and buffalo (0.015 mM), 2 ìl of template DNA (50 ng), 2.5 µl of 10X PCR buffer, 0.06 ìl *TaqDNA polymerase* (0.3 Units) 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 30s at 94°C, annealing at 60°C for 45s, extension at 72°C for 45s and a final extension at 72°C for 10 min. The PCR products were electrophoresed at 85 V for 2 hours in 2% agarose gels after the wells were charged with 5ìl of DNA preparations mixed with 1ìl 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 OD ratio of the extracted DNA from uncooked *Rista* was highest for cattle (1.88) and lowest for buffalo (1.70) with the values of rest of the test samples falling in between the two (Table 3). The data on concentration of extracted DNA from uncooked *Rista* indicated that the values (ìg/ml) ranged from a highest of 1975.00 for T_2 to the lowest of 1725.00 for cattle and T_4. The data of the above referred to parameters indicated that, on an overall basis, the quality of DNA extracted from various samples of the study was good.

The Multiplex PCR using mitochondrial *cyt b* gene specific primers successfully amplified the *cyt b* gene fragments of sheep, cattle and buffalo in each sample of uncooked *Rista* prepared from mixed meats (Fig. 1). The Multiplex PCR amplified fragments exhibited characteristic band pattern that was as expected for the meats of contributing species. The amplified bands of *cyt b* gene were of the sizes of 124bp, 472bp and 585bp for buffalo, cattle and sheep, respectively. The bands of the PCR products of amplified *cyt b* gene fragments of

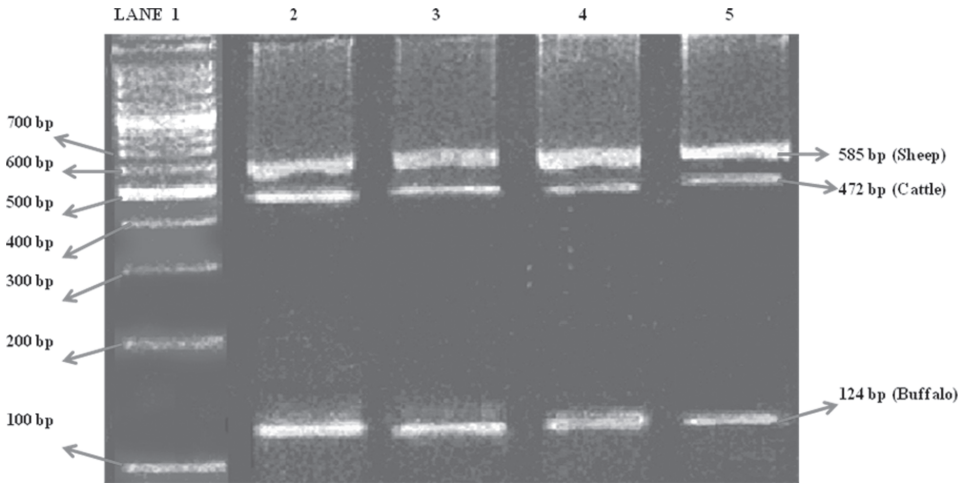

Fig 1. Optimized cyt b Gene Fragment Amplification of Sheep, Cattle and Buffalo from Uncooked Ristaemulsion Prepared from mixed Meats
Lane 1: DNA ladder of 100bp Lane 2-5: Mixed meat (mutton:cattle:buffalo meat in the ratio of 60:20:20, 80:10:10, 90:05:05 and 98:01:01 respectively)

sheep, cattle and buffalo species from uncooked *Rista* prepared from various ratios of mixed meats showing three bands representing the respective three species are presented in Lane 2, 3, 4 and 5. The bands representing mutton of the adulterated emulsion samples were also of good intensities. However, the band intensities for the cattle and buffalo *cyt b* gene fragments showed progressive decline with the reduction of their meat proportion from 20% to 1% in the emulsion. Although, bands representing the adulterant meat (cattle and buffalo meat) showed decline in band intensities with the reduction of adulteration level, but all these bands were visible. Moreover, it was possible to detect cattle and buffalo meat in the mutton Kebab emulsion up to 1% adulteration level. Further, the processing involved in the preparation of *Rista* had no significant effect on meat speciation even up to 1% level.

The results of the present study are in accordance with the reports of earlier workers. Meyer *et al.* (18) has reported the detection of meat species in adulterated mixed meats by tracing 0.5% pork in meat mixture using the duplex PCR technique. Likewise, detection of 1% chicken in mutton using PCR-RFLP (24), 1% pork in cattle labeled meat (11) and 1% chicken meat in meat products by PCR assay (19) has been reported. 0.005% pork in cattle and 1% pork in duck patties by PCR in heated and unheated meats, sausages, canned food, and cured products have been traced (4). Moreover, Calvo *et al.* (25) detected 0.01% uncooked cattle in pork in uncooked emulsion mixture and 1% cattle in cooked meat mixtures by PCR. Similarly, detection of 1% of cattle, sheep meat, goat meat and pork in uncooked and heat treated meat mixtures by PCR assay has been reported (26).

The results of the present study proved the efficiency of mtDNA based test in detecting the meat adulteration even at lower levels, thus making the PCR as a reliable method in the identification of meat species. Among different approaches of PCR, Multiplex PCR is more beneficial for meat speciation because it is highly repeatable, time saving and affordable than the other approaches (27). Among the DNA based techniques, the mtDNA techniques are more advantageous because of the presence of

Table 1. Proportions of meat and fat used in the formulation for *Rista*

Percent Ingredients	Treatments			
	T_1	T_2	T_3	T_4
Mutton	48 (60)	64 (80)	72 (90)	78.40 (98)
Cattle	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)
Cattlefat	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 *Rista* with mutton, cattleand 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

Table 3. Quality of Harvested DNA from Uncooked *Rista*

Source of DNA Sample	OD_{260}nm	OD_{280}nm	OD_{260}nm / OD_{280}nm	Concentration (ig/ml)	Quality of DNA
Mutton	0.351	0.204	1.72	1755.00	Good
Cattle	0.345	0.183	1.88	1725.00	Good
Buffalo	0.370	0.217	1.70	1850.00	Good
T_1	0.377	0.204	1.84	1885.00	Good
T_2	0.395	0.212	1.86	1975.00	Good
T_3	0.388	0.210	1.84	1940.00	Good
T_4	0.345	0.191	1.80	1725.00	Good

T_1: Mutton:cattle:buffalo in the ratio of 60:20:20 T_2: Mutton:cattle:buffalo in the ratio of 80:10:10
T_3: Mutton:cattle:buffalo in the ratio of 90:5:5 T_4: Mutton:cattle:buffalo in the ratio of 98:1:1.

thousands of copies of mtDNA per cell that ensures a sufficiently large quantity of PCR product even in case of samples undergoing intense DNA fragmentation (28). Further, the chances of mtDNA degradation under different meat processing conditions are lesser, thus making it ideal for processed meat species identification (11).

Conclusion

Therefore, in conclusion the mtDNA based multiplex PCR procedure has the ability to detect

up to as low as 1% level of meat adulteration in uncooked mutton rista, thus making it a method of choice in identification of meat species in adulterated meat.

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