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A PCR Assay for Identification of Buffalo Origin of Tissue by Amplification of the mt. D-loop Gene

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ABSTRACT

The present study was carried out with aim to develop and standardize the protocol for species-specific PCR assay for detection of tissue of buffalo origin. Muscle tissue samples from viz: cattle (postmortem), buffalo, sheep, goat and pig were used to extract the DNA and the good quality DNA samples having $OD_{260:280}$ of 1.8-2.1 were used in this study. Species-specific primers for buffalo was designed through homology comparisons of the mitochondrial gene regions from these species using Megalign (DNA- STAR) and designed primer pairs were tested for their specificity by BLAST analysis. The PCR conditions were optimized in terms quantity and concentration of various components for PCR mix and annealing temperature. The developed assay was evaluated for its species specificity and sensitivity. Efficacy and reliability of developed assay was also validated on known samples, samples from meat admixture and samples subjected to diverse heat treatment viz: boiling, autoclave and microwave. The developed species-specific PCR assay resulted in amplification of DNA template exclusively from buffalo samples and resulted in amplified PCR product of 742bp. Sensitivity of the assay was determined by making 10-fold serial dilution of genomic DNA, which showed that only 10ng of absolute DNA content, was required for PCR amplification and successful identification of tissue of buffalo origin. Thus, it was concluded that developed species-specific PCR assay is effective in identification of tissue of buffalo origin.

Keywords: Species specific. PCR assay, mt. D-Loop gene, Buffalo

The demand for buffalo meat is comparatively higher because of inherent low fat, cholesterol and their leanness making it a choice for health conscious people (Giuffrida Mendoza *et al.,* 2015). It has gained increased popularity in several south eastern and middle-eastern Asian countries, a preferred destination for Indian Buffalo meat. Cases of misrepresentation and adulteration in meat and meat products by meat traders are being reported from across the world (Spink and Moyer, 2011; Grundy *et al.,* 2012). In India also, with inception of Food Safety Standard Authority of India, laws pertaining to adulteration, substitution or other kind of falsification in food commodities including meat have become quiet stringent. Authentication of meat is essential because of economic, religious and health implications (Zade, 2002) and to enforce the fair trade in meat industries.

Various authentication investigation reports for meat and meat products carried out by legal authorities across the world reveals that, species substitution is one of the major routine malpractices employed in meat industry by meat sellers (Ayaz *et al.,* 2006). Till date various techniques have been evolved for meat species identification. DNA-based methods especially PCR based techniques have emerged as most appropriate tools for species authentication and have advantages such as the relatively high stability of DNA (Arslan *et al.,* 2006) and conserved nature of DNA within individuals of same species (Girish *et al.,* 2004; Saini *et al.,* 2007). Various versions of PCR assays including species-specific PCR (Arun *et al.,* 2012), PCR-RFLP (Aida *et al.,* 2007), Multiplex PCR Technique (Zhang, 2013), RAPD-PCR Technique (Mane *et al.,* 2006), Real Time PCR Assay (Rojas *et al.,* 2010) and Sequence

Analysis of PCR assay (Martin *et al.,* 2007) have been documented for authentication of meat species.

Species-specific PCR assay seems to be simple and easy method in comparison to others. In this technique, carefully designed species-specific primer set under optimized conditions is conclusive to detect and identify species of interest, eliminating the need of restriction digestion and/or sequencing of PCR products, reducing the cost and labor of assay. Species-specific PCR assay is being useful for routine analysis of large numbers of samples, even when aggressive processing treatment have been applied on meat (Mafra *et al*., 2008 and Rojas *et al,*. 2009). Most of the work on species detection by species-specific PCR assay has been done by targeting mitochondrial DNA due to their pattern of maternal inheritance, absence of sequence ambiguity and higher number of copies per cells (Bellagamba *et al.,* 2001) which increases the probability of positive results even in highly fragmented DNA (Mane *et al.,* 2011).

The existence of buffalo is restricted in geographical area particularly Asian countries like India, Pakistan and Bangladesh has led to limited research on identification meat of buffalo origin. The increase in demand for meat of buffalo origin as compared to beef has drawn the attention of researcher towards development of PCR based assay for authentication of meat of buffalo origin. India, the leading exporter country for buffalo meat, the demand for such an assay by traders is quiet high to safeguard their business interest. In this research paper, a species-specific PCR assay has been presented for successful identification of buffalo meat and its differentiation from meat of others common animals like cattle, sheep, goat and pig. The assay was also helpful in identification of meat species origin in heat processed meat samples.

MATERIALS AND METHODS

Sample collection

The fresh meat samples of buffalo were collected under aseptic condition from the municipal abattoir of Bareilly (UP), and cattle tissue samples were collected from post-mortem hall of IVRI (UP). Meat samples of other animals were collected from experimental abattoir of LPT division IVRI (Sheep, Goat and Pig). The meat samples were collected in a pre-sterilized plastic containers and transported to laboratory under ice-chilled condition (4^{o}C) and stored frozen at -20^{o}C until DNA extractions.

DNA extraction

From tissue samples

High quality genomic DNA were extracted from tissue samples by using *DNeasy Blood and Tissue Kit* (Qiagen, Germany) as per the manufacturer's instructions. 25mg of muscle tissue were taken from samples for DNA extraction. Firstly the sample was grinded with micro pestle in a 1.5 ml micro-centrifuge tube followed by the addition of 20 µl of proteinase K and 180 µl buffer ATL (for lyses tissues). The mixture was incubated at 56^{o}C for 3 hours to lyse the sample. Subsequent steps followed the instructions given by the kit user manual. Quality of extracted genomic DNA was assured by horizontal submarine agarose gel electrophoresis using 0.8% agarose gel. Purity and concentration of DNA was determined by spectrophotometer.

From heat processed tissues sample

The meat samples were cut into slices and processed with different time temperature combination. Three heat treatments were employed viz., boiling water for 30 min, autoclave at 121˚C for 20 min and microwave cooking for 3 min leading to minimal core temperature of 70˚C. Processed tissue samples were cooled to room temperature and DNA was extracted by using same kit. The quality evaluation of DNA was done as stated above.

Design of species-specific primer set

Species-specific primer was designed by targeting mitochondrial D-loop gene of buffalo. The published DNA sequences of all other mentioned species intended for these genes were retrieved from National Center for Biotechnology Information (NCBI) Gene Bank and were aligned using megalign software to select the inter-species hyper-variable regions. These hyper variable regions were selected and used to design buffalo specific primer set using primer designing soft-ware (DNA-STAR Inc., USA). The details of primer set used in the present investigation are shown in table 1 as below:

Table 1: Details of Species-specific primer set used for identification of tissue of buffalo origin

Species	Gene targeted	Primer code	Type	Nucleotide sequence 5'----------3'	Product length
Buffalo	D-loop	LPTM/ D-loop -(B)BF	Forward	AGAAATAACTACAACCATCAACACACC	742
		LPTM/ D-loop -(III)BR	Reverse	GAATAAGCATCTAGGGAGAAGCATGT	

The designed primer set was also screened for unique specificity to eliminate the possibility of cross-species binding using online BLAST local alignment tool in NCBI data bases (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). The designed primer set was custom synthesized and procured from Eurofins Genomic India Pvt. Ltd (Banglore).

Optimization of PCR conditions

Preliminary trials were conducted to standardize the quantity and concentration of various components for PCR mix. The annealing temperature for designed primer pairs were optimized by PCR amplification of DNA templates of buffalo origin using gradient PCR set to with temperature range 54-64°C.

Buffalo species specific PCR amplification

Good quality extracted from tissue samples of cattle, buffalo, sheep, goat and pig were subjected to PCR amplification using designed primer set. The reaction mixture was prepared in a final volume of 25 µl in a 200 µl PCR tube (Tarson). Quantity and concentration of various components used for PCR amplification were as per Table 2. The thermal cycling protocol of PCR amplification for DNA template is shown in Table 3.

Gel electrophoresis of Amplified PCR products

Horizontal submarine agarose gel electrophoresis was used. 1.5% agarose (w/v) suspension in 0.5X TBE buffer was made and heated on an electric oven until the agarose was completely melted to give a clear transparent solution. After cooling to 60°C, ethidium bromide (10 mg/ml) @ 5 µl per 100 ml of agarose solution was added and mixed gently. The agarose was poured into a levelled casting tray and the gel was made to about 4 mm in thickness. The agarose was allowed to set at room temperature till it solidified and subsequently the comb was gently removed. 5 µl of PCR products were mixed with 2 µl of 6 X gel loading dye, loaded into the well of agarose gel and a 100 bp DNA ladder (Fermentas) was used as size marker and run simultaneously in all the runs. Electrophoresis was performed at 110V for 30-45 min. Once the electrophoresis was over, the gel was visualized under UV transilluminator and documented under gel documentation system (AlphaImager HP, California, USA).

Table 2: Quantity and concentration of various components used in PCR master mix

Sl. No.	Components	Quantity (µl)
1	10X Dream Taq Buffer	2.5
2	d-NTP Mix 10mM each	0.5
3	Dream Taq Polymerase(5U/ µl)	0.2
4	Forward Primer (10pM/ µl.)	1.0
5	Reverse Primer(10pM/ µl.)	1.0
6	Nuclease Free Water	18.8
7	DNA Templet (50 ng/µl)	1.0
	Total	25.0

Table 3: Thermal cycling protocol for PCR

Sl. No.	Cycling Steps	Protocol	
		Temp.	Time
1	Hot Start	95 ˚C	5min
2	35 cycle of Denaturation	95 ˚C	60sec
	Annealing	AT	45sec
	Extention	72 ˚C	45sec
3	Final Extention	72 ˚C	10min
4	Storage	4 ˚C	α

Determination of sensitivity of PCR assays

The sensitivity of developed PCR assays were determined by making 10 fold serial dilution of buffalo DNA template pre-adjusted to stock concentration of 50ng/µl. The PCR amplification pattern was observed for each diluted DNA sample.

Laboratory validation of PCR assays

Efficacy and reliability of developed PCR assays were validated on DNA samples obtained from ten different individuals of buffalo. The utility of these species-specific primer sets were also verified by identification of buffalo origin of meat in meat admixture. Following two sets of meat admixtures were prepared by mixing the equal amount of chopped tissues from species as indicated below

a. Cattle, buffalo. sheep, goat and pig

b. Cattle. sheep, goat and pig

The genomic DNA was isolated from these meat admixtures by using *DNeasy Blood and Tissue Kit* (Qiagen, Germany) as per the manufacturer's instructions. The PCR amplifications were carried out using optimized protocol.

DNA extracted from heat treated tissue samples were also subjected to PCR amplification using designed primer set.

RESULTS

The $OD_{260:280}$ ratios of extracted DNA either from tissue sample, heat processed samples or meat admixture were found to be in range of 1.8-2.1 which indicated high quality of DNA in all samples. The concentration of genomic DNA ranged between 30-65 ng/ µl and showed clear smear pattern on gel electrophoresis. The observed pattern of amplifications for primer set LPTM/ D-loop-(B)BF & LPTM/ D-loop- (III)BR revealed that 62˚C was the optimum annealing temperature for the primer set.

The electrophoretic pattern of buffalo specific amplification is shown in Fig. 1. The PCR amplification carried out using designed primer sets was found to be very species-specific. It successfully amplified the buffalo DNA and not the DNA from other species. The buffalo specific primer sets LPTM/ D-loop- (B)BF & LPTM/ D-loop- (III)BR amplified the template DNA to amplicon size of 742 bp. No amplifications were observed with sample having DNA of cattle, sheep, goat and pig. T h e electrophoretic pattern of PCR amplification DNA from of heat-treated tissue samples is shown in . 2. It showed

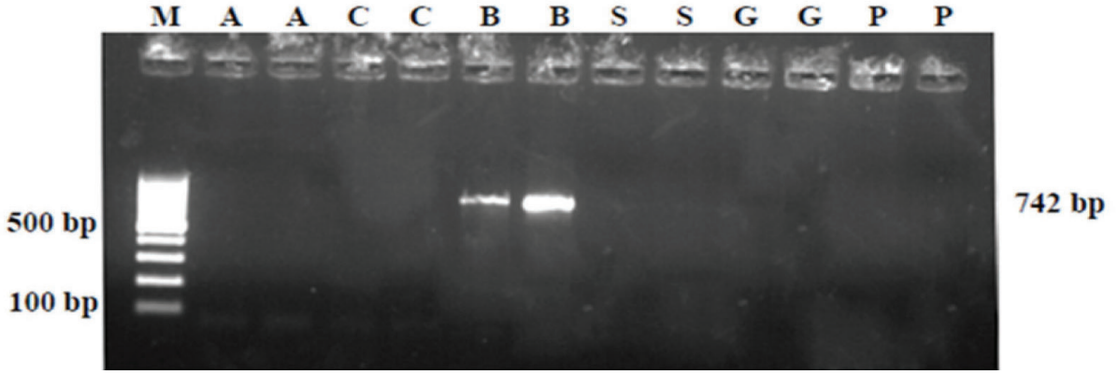

Fig. 1: Specificity of buffalo specific mt. D-loop primer: Electrophoretic pattern of amplified product (742 bp) from PCR using LPTM D-loop (B)BF & (III) BR primer set. Lane M: 100 bp ladder (Fermentas), Lane 1: Negative control; Lane C: Cattle; Lane B: Buffalo; Lane S: Sheep; Lane G: Goat; Lane P: Pig

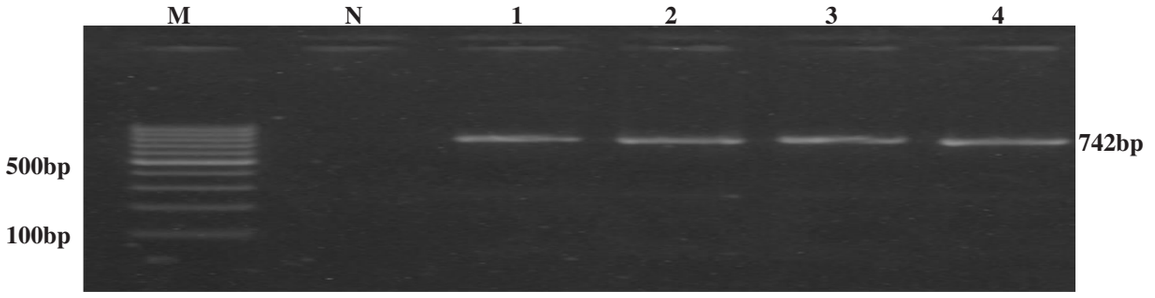

Fig. 2: Applicability of buffalo specific mt. D-loop primer in heat treated tissue samples: Electrophoretic pattern of amplified product (742 bp) from using LPTM/D-loop(B)BF & (III) BR primer set. Lane M: 100 bp ladder (Fermentas); Lane 1: Negative control; Lane B: Unprocessed buffalo tissue; Lane 1: Boiling water for 30 min.; Lane 2: Autoclave at 121°C; Lane 3: Microwave cooking for 3 min.

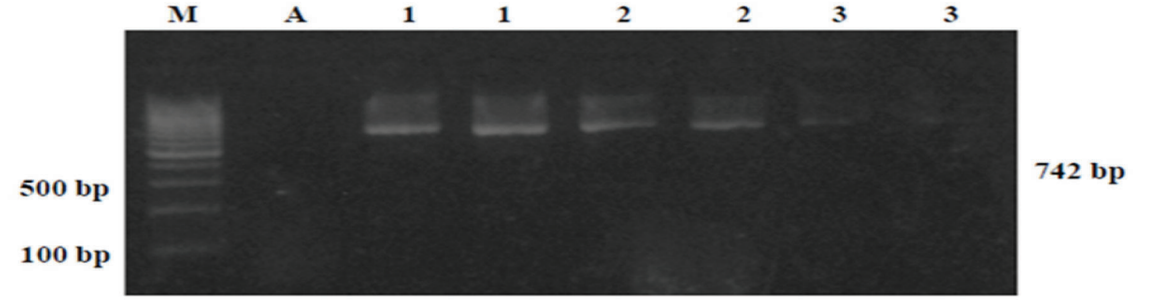

Fig. 3: Sensitivity of buffalo specific mt. D-loop primer: Electrophoretic pattern of amplified product (742 bp) from using LPTM/D-loop(B)BF & (III) BR primer set. Lane M: 100 bp ladder (Fermentas); Lane A: Negative control; Lane 1: 100ng genomic DNA; Lane 2: 10ng genomic DNA; Lane 3: 1ng genomic DNA.

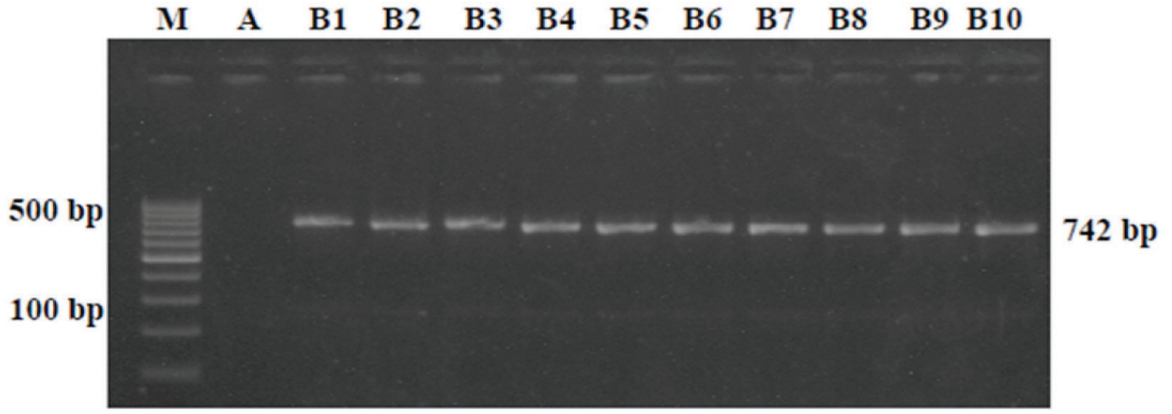

Fig. 4: Laboratory validation of the assay in known samples using buffalo specific mt. D-loop primer : Electrophoretic pattern of amplified products (742 bp) from PCR using LPTM/D-loop(B)BF & (III)BR primer set. Lane M: 100 bp ladder(Fermentas); Lanes A: Negative control; Lane B1-B10: DNA samples from 10 different buffalo.

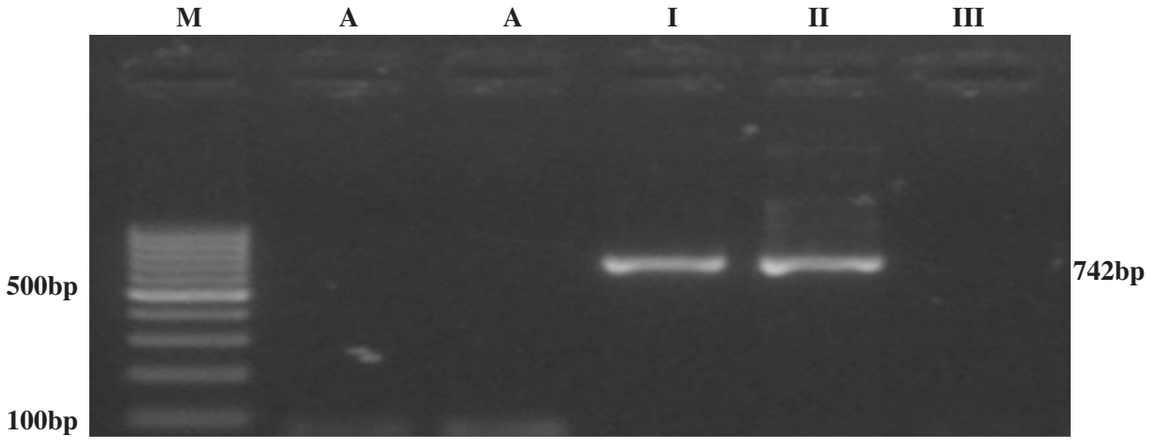

Fig. 5: Laboratory validation of the assay using buffalo specific mt. D-loop primer on meat admixture samples: Electrophoretic pattern of amplified products (742 bp) from PCR using LPTM/D-loop(B)BF&(III)BR primer set. Lane M: 100 bp ladder(Fermentas); Lanes A: Negative control; Lane I: Positive control (Buffalo); II: Meat admixtures- Cattle, Buffalo, Sheep, Goat and Pig; III: Cattle, Sheep, Goat and Pig

that the specifically designed primer sets also successfully amplified the DNA extracted from tissues subjected to various heat treatments.

The electrophoretic pattern of sensitivity results of buffalo specific PCR assays with mitochondrial D- loop primers set LPTM/ D-loop- (B)BF & LPTM/ D-loop- (III) BR is shown in Fig. 3. It is evident from the figure that lowest absolute amount of DNA template which could be amplified was 10ng. The diluted template below this concentration could not be amplified by primer sets.

DNA isolated from samples of 10 different individuals of buffalo was subjected to PCR amplification with designed species-specific primer sets. The primer pairs amplified the template DNA of all the 10 individuals of buffalo included in study to corresponding fragment length of 742 bp (Fig. 4).The electrophoretic patterns of amplification of template DNA from meat admixture of cattle, buffalo, sheep, goat and pig with the fragment length of 742 bp revealed the ability of developed assay to amplify the DNA from meat admixture under optimized PCR conditions (Fig. 5).

DISCUSSION

The present study was carried out with the aim to develop an easy and simple technique for identifying buffalo origin of tissue. Over the last two decades, DNA based PCR assays have raised the hope for developing authentic and robust method for species identification in meat sample to trace the species origin in meat food. The designed species-specific primer sets targeted the intra-species conserved and inter-species hyper variable regions of mitochondrial D-loop. The primer design is very crucial step in species-specific PCR development, it must contain adequate conserved sequence within species, and SNPs in 3' end of primers between species. (Ali *et al*., 2015) Compared to the methods targeting genomic DNA, PCR amplification of mitochondrial DNA is extensively beneficial in species determination and allows low limit of detection due to the numerous mitochondria in most cells (Yang *et al.,* 2014). Detection of carabeef by species-specific PCR has been reported previously by targeting nuclear (Guoli *et al.,* 1999) as well as mitochondrial DNA sequences (Malisa *et al.,* 2006) but their primer sequences were different.

The specificity of the PCR was evaluated against species viz: cattle, sheep, goat and pig and found to be specific for buffalo only, indicative of designing of primer from hyper variable region. Most of the work on species detection by species-specific PCR has been done by targeting mitochondrial genes such as Cyt.B, 12S rRNA, 16S rRNA, ND5 and D-loop regions. For identification of buffalo meat, Narendra *et al*. (2014) designed a specific oligonucleotide by targeting the conserve region of Cyt.B gene.

Species identification of cooked meat is often necessary. The three different type of heat treatment viz: Boiling, Autoclave and Microwave cooking included in this study were commonly practiced cooking methodology. Identification of buffalo origin from DNA obtained from cooked samples is one of the very important merits of this technique. A rapid and highly specific assay for detection of turkey and chicken in processed meat products was reported by Hird *et al.* (2003) and was based on PCR amplification of species-specific amplicons with rapid visualization using vistra green.

Levels of DNA lower than 10ng in the PCR mixture were not amplified clearly to be visible in the gel electrophoresis system. Even lower concentration of DNA has been reported to be amplified with species-specific primer sets. Dai *et al.* (2015) reported the sensitivity of the conventional PCR assay was down to 0.001 ng of DNA template. In our case mt. genome was targeted which might have been proportionally lower in used DNA template because of sampling of tissue from muscle with less number of mitochondria in their cells. Miller *et al*., 2003 has also cited lower copy number of mt DNA per diploid nuclear genome in skeletal muscle as compared to myocardium.

The finding reveals the suitability of primer set for identification of buffalo tissue in meat admixture, which is one of the most common frauds. It also indicated that the detectable amount of target DNA could be isolated and identified in case of partial substitution or mixing of meat samples. Bhat *et al.* (2016) conducted an experiment for detection of meat species in admixture and found that PCR procedure efficiently detected falsification of cooked mutton product with beef and buffalo meat up to 1% level. In our study only qualitative analysis was done.

CONCLUSION

In the present study the buffalo specific PCR assay were found to be very species –specific, sensitive, reliable and accurate in the identification of buffalo meat even in heat processed and meat admixture of different meat species. The present work would help in addressing social, religious, economic, public health issues and forensic related to buffalo meat species identification and, further, it could also be used for routine analysis of suspected meat samples.

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