

IDENTIFICATION OF BUFFALO (*Bubalus bubalis*) MEAT
USING PCR TARGETING MITOCHONDRIAL D-LOOP GENE

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ABSTRACT

A novel polymerase chain reaction (PCR) was developed for the identification of buffalo (*Bubalus bubalis*) meat using newly designed primers targeting the mitochondrial D-loop region. Buffalo-specific primers were designed against a conserved region of mitochondrial d-loop that amplified buffalo specific region of 358 bp in size. The specificity of primers was confirmed by PCR analysis of DNA from related domestic animal meats i.e. cattle, goat, sheep, pig and chicken. The PCR assay was checked for repeatability using DNA isolated from different buffalo meat samples and was validated. Buffalo species-specific PCR developed in this study presents a means of identification of buffalo meat as a reliable tool to avoid the fraudulent substitution and adulteration of buffalo meat.

Keywords: meat, adulteration, buffalo, d-loop, PCR

INTRODUCTION

In India, there are two major factors associated with the consumption of buffalo meat (carabeef). Firstly, the Hindus have reservation towards the consumption of buffalo meat, while the

Muslim community prefers to consume carabeef in view of ban on the slaughter of the cows (beef) in this country. Secondly, there is a malpractice among meat vendors to mix the low priced carabeef (even sometimes the banned cow meat) meat with other costlier meats like goat (chevon) and sheep (mutton) meats to gain monetary benefits. Under such circumstances, the consumers would have questions pertaining to the surety and authenticity of the origin of meat. Also, every year a huge number of veterolegal cases are registered involving buffalo killing in India including the wild ones. Keeping in view these peculiarities in India, carabeef identification has become an essential element for food quality control and forensic analysis. A number of analytical procedures have been evolved to correctly differentiate various food animal species. Most of analytical methods employed are based on the protein analysis by either electrophoretic (Vallejo *et al.*, 2005), chromatographic (Toorop *et al.*, 1997), or immunochemical assays (Chen and Hsieh, 2000). However, most proteins get denatured at high temperatures, resulting in changed antigenicity and electrophoretic mobility of molecules (Giovannacci *et al.*, 2004). Recently, DNA-based methods particularly polymerase chain reaction (PCR) has proved to be a reliable tool for rapid detection and identification of organisms at the species level. Using an appropriate primer pair, mitochondrial sequences have been amplified in

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many species, and the resulting differences used for species identification (Di Pinto, *et al.*, 2005).

Several mitochondrial genes including cytochrome-b gene (Forrest and Carnegie 1994; Verma and Singh, 2003), the 12S and 16S ribosomal RNA genes (Rodríguez *et al.*, 2003; Fajardo *et al.*, 2006), and the displacement loop gene (d-loop) (Lopez *et al.*, 1996; Gao *et al.*, 2004; Kierstein *et al.*, 2004) have been targeted for species identification. The D-loop is the most rapidly evolving region of the mt DNA molecule and is one of the most commonly used markers (Kocher *et al.*, 1989; Foran *et al.*, 1997) when determining evolutionary relationships among closely related species and subspecies. Keeping in mind the need for a reliable technique for identification of buffalo meat, the present study was aimed to develop a buffalo specific PCR assay for the authentic identification of buffalo meat (carabeef).

MATERIALS AND METHODS

1. Meat samples

Meat samples from buffalo (*Bubalus bubalis*), cattle (*Bos indicus*), goat (*Capra hircus*), sheep (*Ovis aries*), pig (*Sus domesticus*) and chicken (*Galus gallus*) were used in the present study. Cattle, goat, sheep, pig and chicken meats were used to check the specificity of the designed primers. Approximately, 50 gm of meat samples were collected from local markets, slaughter houses and veterinary clinics under sterile conditions and were transported to laboratory in an icebox containing gel cool packs. Meat samples were kept in deep freezer maintained at 20 °C till further use.

2. DNA Extraction

The DNA was isolated from the samples

using a Wizard® Genomic DNA purification kit (Promega, Madison, USA) following the manufacturer's instructions. Purity, quality and concentration were determined as per standard protocols.

3. Designing of primers

Buffalo specific primers were designed targeting mitochondrial d-loop (DNASar, Inc., 1996). The buffalo mitochondrial d-loop sequences were downloaded from the NCBI and aligned using "Megalign" software (DNASar, Inc., 1996). A conserved region was identified and oligonucleotide primers were designed using "Primer-Select" software (DNASar Inc., 1996) so as to yield a PCR product of 358 bp specific for buffalo. Later, the selected primers were confirmed for specificity by using the PRIMER-BLAST of NCBI. Finally, selected primers were custom synthesized from IDT, USA and used for PCR amplification. The primer sequences were forward (DAF-01, 5'-TTCTTCAGGGCCATCTCATC-3') and reverse (DBR-03, 5'-TCGAATAAGCATC TAGGGAGAA-3').

4. Standardization of PCR

The PCR conditions were standardized so as to obtain the desired amplicon of 358 bp for buffalo. A 25 µl reaction mixture was prepared containing 2.5 µl of 10X assay buffer [25 mM MgCl₂, Bioron, GmbH], 0.5 µl (200 µM each) of dNTP mix [sodium salts of dATP, dCTP, dGTP and dTTP 10 mM each in water i.e., 40 mM total, pH 7.5, Promega, USA], 0.8 µl (20 Pico moles) each of forward and reverse primers (Integrated DNA Technologies - IDT, Madison, USA), 1U Taq DNA polymerase (DFS-Taq DNA polymerase, Bioron, Germany), 50 ng of purified DNA and nuclease free water (Merck, Germany) to make the volume. The

tubes were flash spun and the PCR was performed in a Thermal cycler (Gene AMP® PCR System 9700, Applied Biosystems).

The cycling conditions were as follows: an initial denaturation (95°C for 5 minutes) followed by 30 cycles of denaturation (95°C, 30 seconds), primer annealing (52°C, 30 seconds) and extension (72°C, 30 seconds) and final extension (95°C, 5 minutes). The PCR products were held at 4°C until electrophoresis. Agarose gel (2%) was prepared in 0.5X TBE buffer and the PCR products (8 µl) stained with 6X gel loading dye (2 µl) were electrophoresed at 50V for 1.5 h along with 100 bp DNA ladder (M/s. Bangalore Genei, India). The amplified products were visualized using a gel documentation system (AlphaImager® HP, Alpha Innotech Corp.).

RESULTS AND DISCUSSION

Fraudulent substitution of buffalo meat with other costlier meats demands the development of simple and authentic method for detecting buffalo meat. The present investigation was undertaken with the objective to develop a simple and specific PCR based molecular diagnostic techniques for the identification of buffalo meat.

1. Standardization of buffalo specific PCR assay

A fragment of 358 bp from the targeted buffalo mitochondrial D-loop region (Accession no. AF197216, location 490-847) was amplified (Figure 1). Primer concentration of 20 pico moles per reaction and an annealing temperature of 52°C were found ideal for amplification. Different primer

concentrations ranging from 18-22 pico moles were attempted to obtain the desired PCR product of 358 bp and finally a primer concentration of 20 pico moles was selected for amplification. Similarly, different annealing temperatures ranging from 48-58°C were used for the standardization and lastly 52°C was selected as an optimum annealing temperature. In a similar study, Malisa *et al.* (2006) reported PCR amplification of mitochondrial D-loop for identification of buffalo meat species but with different primer sequences. This study is in accordance with the work done by Nagappa (2008), who differentiated six food animal species including buffalo, targeting mitochondrial D-loop with species-specific primers; and who also employed species-specific PCR assays for the detection of origin of meat species in raw, heat treated as well as adulterated meat samples. Similarly, Guoli *et al.* (1999) reported PCR amplification of a 218 bp product specific for buffalo DNA by targeting 1.709 satellite DNA.

2. Specificity and repeatability of standardized PCR assay

Possibility of cross amplification of buffalo specific primers was eliminated by testing buffalo specific primers with DNA of cattle, goat, sheep, pig and chicken. The buffalo specific primer pair was able to produce amplicon of 358 bp in buffalo DNA only (Figure 2). No amplification was observed in the DNA of other species tested including negative control and thus the specificity of designed buffalo specific primers was confirmed. Repeatability of buffalo specific primers was confirmed by testing primers with DNA isolated from different buffalo meat samples (5 each) collected from different places. Invariably, the amplicon of 358 bp specific for buffalo DNA was obtained (Figure 3).

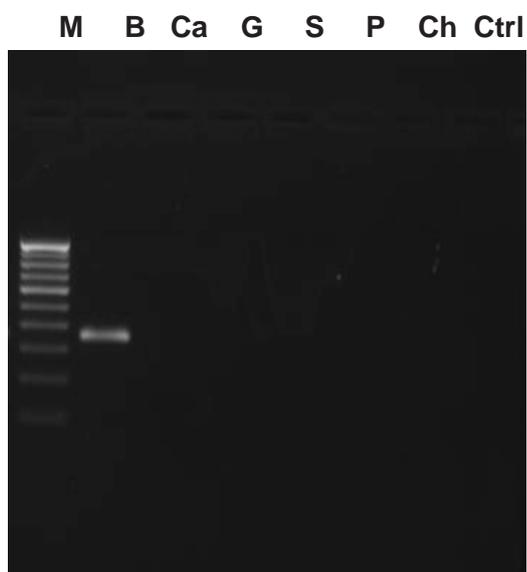


Figure 2. PCR amplification of buffalo DNA (358 bp). M-100bp DNA marker, B-Buffalo, Ca-Cattle, G-Goat, S-Sheep, P-Pig, Ch-Chicken and Ctrl-Negative control.

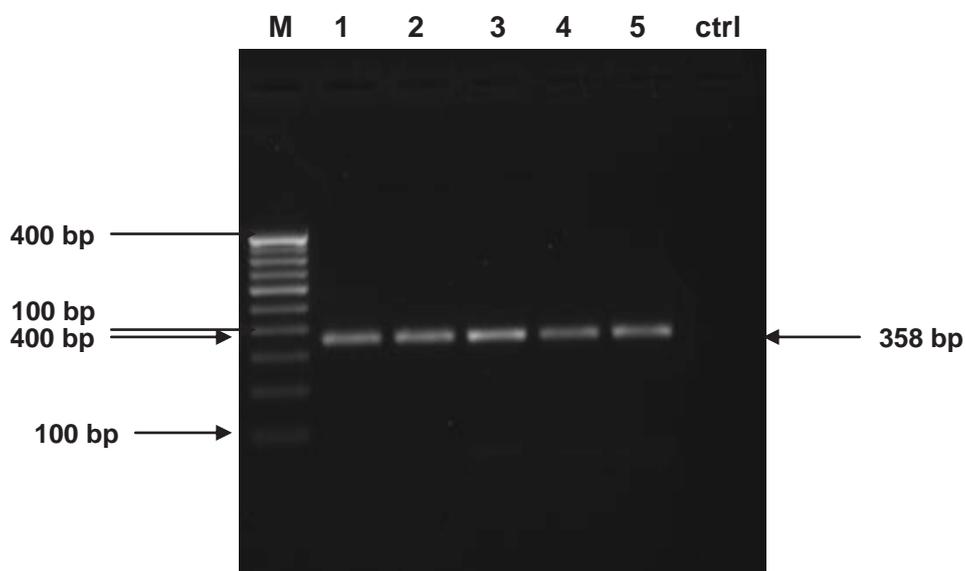


Figure 3. PCR amplification of buffalo DNA (358 bp). M-100bp DNA marker, 1-5 (buffalo meat samples) and Ctrl-Negative control.

CONCLUSION

The conventional methods available for meat species identification lack specificity and repeatability. To overcome these problems DNA based techniques are employed for species identification. Specific PCR assay was developed for identification of buffalo meat by amplifying a conserved region of mitochondrial D - loop gene. The assay was found to be highly specific. The single step PCR assay developed for identification of buffalo meat presents a reliable tool to solve adulteration, falsification and veterolegal problems related to buffalo meat.

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