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DIFFERENTIATION OF CATTLE AND BUFFALO MEAT USING MULTIPLEX POLYMERASE CHAIN REACTION

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ABSTRACT

Authentication of origin of animal species is of paramount importance owing to economic, social, religious, forensic and public health considerations. In the present study, a multiplex polymerase chain reaction (mPCR) was developed by designing unique species-specific forward and a common reverse primers flanking mitochondrial D-loop region for the authentication of cattle (*Bos indicus / B. taurus*) and buffalo (*Bubalus bubalis*) species. Novel mPCR amplified 382 bp product specific to cattle and 336 bp specific to buffalo. Possibility of cross amplification in other species was rules out by testing as many as 25 other animal species. The novel mPCR assay developed in this study for the authentic identification and differentiation of meats of cattle and buffalo could be applied for the differentiation of samples derived from cattle and buffalo in diagnostic laboratories thereby ensuring prompt labelling of products, protecting consumer sentiments and enforcement of laws.

Keywords: *Bubalus bubalis*, buffaloes, adulteration, labelling, mitochondrial D loop, primer, speciation

INTRODUCTION

Authentication of meats different animal species is of paramount importance owing to economic, social, religious, forensic, and public health considerations. Specifically in Indian subcontinent, identification of cattle and buffalo meat is of utmost importance as slaughter of cow is banned in many states due to religious beliefs; slaughter of both male and female cattle is banned in few states, whereas, very limited restrictions are imposed on the slaughter and consumption of buffalo meat. Apart from the domestic consumption, buffalo meat is also exported to over 70 countries and India is the topmost buffalo meat exporting country in the world. India exported 1.24 million tonnes of buffalo meat worth 3,608 million USD in the year 2018-

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2019 (Agricultural and Processed Food Products Export Development Authority, 2019). On the other hand, export of beef (cattle) is completely banned. Nevertheless, illegal misrepresentation of beef in buffalo meat is often reported for economic gains; this deliberate act violates trust and religious sentiments of the people requiring reliable and rapid test for the authentication of buffalo meat and beef so as to ensure that export consignments do not violate the law of the land. In a study, Werfel *et al.* (1997) diagnosed allergic reactions in 11 (3.28%) of 335 atopic children who consumed beef. Further, detection of mammalian tissue in animal feed is also important for the enforcement of food-labelling laws and the prevent spread of diseases such as transmissible spongiform encephalopathies that affect cattle and humans (Prusiner, 1994).

Adulteration of foods of bovine (cattle and buffalo) meat and products has been encountered in a myriad of intricately woven array of domestic and intentional activities. This malicious sullying is fraudulence punishable under the court of law due to social, religious, forensic, and public health implications. Also, buffalo meat is illegally adulterated with the cattle meat and originality is masked at the time of selling. In addition, issues like insurance claims, forensic cases, illegal trade, etc also demand authentic identification and differentiation of origin of cattle and buffalo species.

Previously, several analytical methods based on anatomical, histological, microscopic, organoleptic, chemical, electrophoretic, chromatographic, and immunological characteristics have been used for the purpose of species identification. However, due to certain limitations, these assays were replaced by currently used DNA based methods. The DNA being stable and highly conserved molecule allows discrimination of species even when sample meat is cooked and processed. Moreover, it also helps in discriminating closely related species. Several DNA based techniques have been used for the authentication of species namely DNA Hybridization (Janssen *et al*., 1998), random amplified polymorphic DNA (RAPD) finger printing (Williams *et al*., 1990), polymerase chain reaction (PCR) and its variants (Matsunaga *et al*., 1999; Kumar *et al*., 2011), restriction fragment length polymorphism (RFLP) (Girish *et al*., 2005), terminal restriction fragment length polymorphism (T-RFLP) (Wang *et al*., 2010), PCR-sequencing (Karabasanavar *et al*., 2010), real-time PCR (Sawyer *et al*., 2003), loop mediated isothermal amplification (Girish *et al*., 2020), etc. Among these techniques, PCR based methods offer greatest advantage in terms of sensitivity, specificity and rapidity (Karabasanavar *et al*., 2011a). Particularly, multiplex PCR can be useful tool for the purpose of species identification because it can identify DNA of several species with precision in a single reaction mixture. Keeping in view the need of a rapid and precise technique for identification and differentiation of cattle and buffalo meat, present work was designed to develop a novel multiplex PCR based assay for the unambiguous authentication of these two species.

MATERIALS AND METHODS

Sampling

In the present investigation, authentic meat or blood samples were collected for PCR analysis from species *viz. Bos indicus* and *B.*

taurus), buffalo (*Bubalus bubalis*), goat (*Capra hircus*), sheep (*Ovis aries*), chicken (*Gallus gallus*), pig (*Sus scrofa domesticus*), horse (*Equus caballus*), dog (*Canis familiaris*), rabbit (*Oryctolagus cuniculus*), camel (*Camelus dromedarus*), turkey (*Meleagris gallopavo*), guinea fowl (*Numida meleagris*), duck (*Anas platyrhynchos*), Japanese quail (*Coturnix japonica*), leopard (*Panthera pardus*), tiger (*Panthera tigris*), barking deer (*Muntiacus muntjak*), sika deer (Cervus nippon), goral (*Naemorhedus goral*), kite (*Milvus migrans*), parakeet (*Psittacula krameri*), elephant (*Elephas maximus*), sambar (*Cervus unicolor*), and fish (*Tor putitora, Schizothorax richardsonii, Raiamas bola, Hypophthalmichthys molitrix* and *Garra gotyla gotyla*).

Approximately, 50 g of meat sample was collected from authentic local markets, abattoirs, dairy, and poultry farms. Since cow slaughter is banned in India, tissue samples were collected from surgical cases (excised tissue) brought to the Veterinary Clinic, College of Veterinary and Animal Sciences, Pantnagar, Uttarakhand. Blood samples were collected from wild animals of Pandit G.B. Pant High Altitude Zoo, Nainital, Uttarakhand. Fish samples were collected from the Directorate on Cold Water Fisheries, Bhimtal, Uttarakhand. All samples were transported to the laboratory under chilled conditions (4ºC) and stored at -20ºC until further use.

DNA isolation

DNA was extracted from meat samples using Wizard Genome DNA purification kit (Promega, Madison, USA) as per the manufacturer's protocol. Phenol-chloroform method was also used for blood samples and isolated DNA was checked for its quality, purity, and concentration (Sambrook and Russell 2001); only good quality DNA with known concentration was used as template to PCR.

Designing of multiplex primers

In order to design highly specific multiplex primers, mitochondrial D-loop sequences of cattle and buffalo species were downloaded from the GenBank database and aligned using "*Megalign*" program (Lasergene, DNAStar, Inc. USA). Thereafter, unique sequences having inter-species heterogeneity and intra-species homogeneity were located and oligonucleotide primers were designed against such unique sequences using "*Primer-Select*" program of DNAStar; selected primers were expected to amplify 382 bp and 336 bp amplicons in case of cattle and buffalo, respectively. Chosen primers were screened for species-specificity and possibility of cross-reactivity using local alignment tool BLAST tool (http://www.ncbi.nlm.nih.gov/blast) and custom synthesized (IDT, Inc. Coralville, Iowa, USA). Primers designed and used for mPCR the present study were;

Forward - cattle: 5′-TAT CAA AAA TCC CAA TAA CTC AAC ACA-3′

Forward - buffalo: 5′-TAG AAA TAA CTG CAA CCA TCA ACA C-3′

Common reverse: 5′-GGG CCC GGA GCG AGA AG-3′

Multiplex PCR

Reaction was set up in 25 µl volume containing 2.5 µl of 10X assay buffer (160 m*M* $(NH_4)_2SO_4$, 670 m*M* Tris-HCl, pH 8.8, 0.1% tween-20, 25 m*M* $MgCl_2$, Bioron, GmbH), 0.5 µl (200 µ*M*) 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), 0.5 µl (20

Pico moles) each of forward and reverse primers (IDT, Inc.), 1U Taq DNA polymerase (DFS-Taq DNA polymerase, Bioron, GmbH, Germany), 50 ng of purified DNA and nuclease free water (Merck, Darmstadt, Germany). The PCR cycling parameters were maintained as follows: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of three successive steps: denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds. The final extension was allowed at 72°C for 5 minutes. After the final extension, the PCR products were held at 4°C until electrophoresis. Thereafter, 5 µl amplified PCR products were electrophoresed on agarose gel (2%) at 50 V for 1 h and visualized over a gel documentation system (AlphaImager HP, Alpha Innotech Corp. USA).

Specificity and repeatability of multiplex PCR

The specificity of the multiplex PCR was evaluated by testing primers with DNA from non-target species. Cross-amplification of non-target species was ruled out by considering as many 25 non-target species. In order to assess robustness of the assay, multiplex PCR was repeated 20 times with constant reagents and cycling conditions.

RESULTS AND DISCUSSIONS

Novel multiplex PCR assay targeting mitochondrial D-loop region was developed for the authentic identification of cattle and buffalo species.

mPCR for the simultaneous detection of cattle and buffalo

Developed multiplex PCR assay efficiently differentiated cattle (*Bos indicus/ B. taurus*) and buffalo (*Bubalus bubalis*) DNA (Figure 1). Multiplex primers designed to amplify unique species-specific regions of mitochondrial D-loop region yielded distinct diagnostic PCR products in mPCR *viz*. 382 bp in cattle and 336 bp in buffalo evidenced after agarose gel (2%). electrophoresis (Figure 1). Difference of 46 bp between PCR products of cattle and buffalo species was sufficient for the identification of species.

Primers of cattle (*Bos taurus*/ *Bos indicus*) are located on its mitochondrial sequence (Accession No. MN714217.1) at positions 15844-15870 (cattle-specific forward primer) and 16209-16225 (common reverse primer) resulting in the amplification of 382 bp PCR product (Figure 2A). Likewise, buffalo (*Bubalus bubalis*) primer is located at bubaline mitochondrial sequence (Accession No. MN756622.1) at positions 15534-15558 (buffalo-specific forward primer) and 15853-15869 (common reverse primer) resulting in amplification of 336 bp PCR product (Figure 2B).

Specificity and repeatability

The specificity of multiplex PCR assay was validated by including as many as 25 species; mPCR amplification of diagnostic amplicons of 382 and 336 bp was evident only in cattle and buffalo species; while none of the other species tested revealed any product. These results indicated higher specificity of the mPCR. The mPCR amplification of target DNA using multiplex primers was repeated 20 times

with different samples keeping amplification conditions constant and consistent results were recorded for meat samples.

Chances of cross-amplification were ruled out as evidenced by the absence of amplification in any of the other species (n=25) tested. Primer concentrations and annealing temperatures were standardized for the mPCR; wherein, primer concentration of 20 pico moles and annealing temperature of 55ºC were found optimum for amplification. Different mitochondrial targets have been used for animal speciation *viz*. cytochrome b (Parson *et al*., 2000; Rajapaksha *et al*., 2003), rRNA (5 seconds, 12 seconds, 16 seconds, 18 seconds) genes (Girish *et al*., 2004; Guha and Kashyap, 2005; Martin *et al*., 2007; Karabasanavar *et al*., 2010) and D-loop region (Karabasanavar *et al*., 2011b, c; De *et al*., 2011). Being a hyper variable region, mitochondrial D-loop proved beneficial in designing specific multiplex primers for the discrimination of cattle and buffalo species. In a similar investigation, Takeda *et al*., (1995) reported sequence polymorphisms in mitochondrial D-loop region that aided in identification of different pig breeds.

Several techniques have been used previously for species identification of cattle and buffalo meat namely PCR-RFLP (Girish *et al*., 2005), species-specific PCR (Karabasanavar *et al*., 2011c), RAPD-PCR (Martinez and Yman 1998), multiplex PCR (Matsunaga *et al*., 1999); PCR-sequencing (Karabasanavar *et al*., 2010) and real-time PCR (Brodmann and Moor, 2003); however, these techniques (PCR-RFLP and PCR sequencing) are cumbersome, costly and require

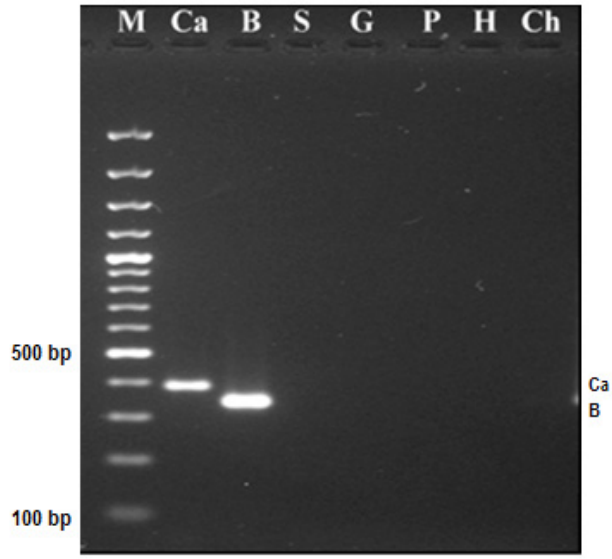
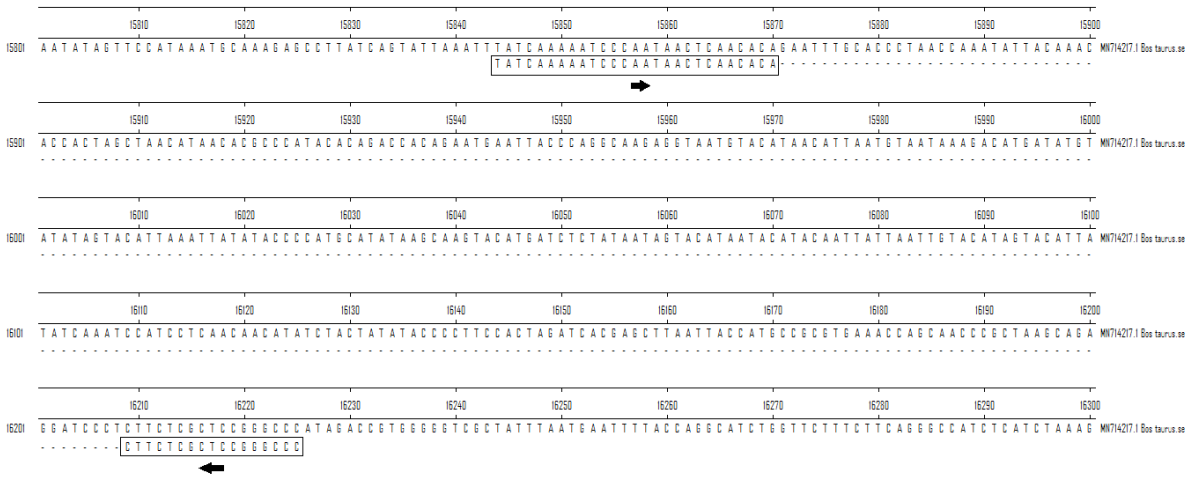
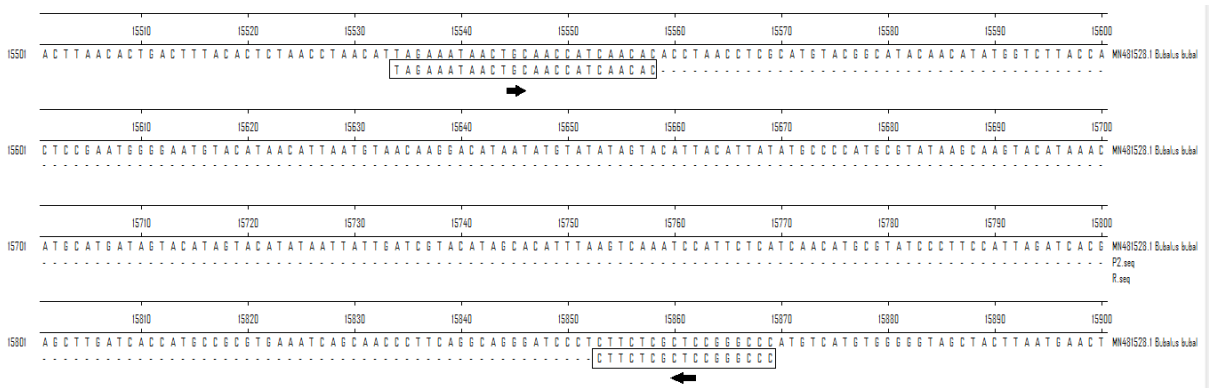


Figure 1. Agarose gel electrophoresis of mPCR products showing cattle and buffalo species-specific amplifications (Lanes M: 100 bp ladder; Ca: cattle; B: buffalo; S: sheep; G: goat; P: pig; H: horse; Ch: chicken).



A) Primer locations for *Bos taurus* / *Bos indicus*



B) Primer locations for *Bubalus bubalis.*

Figure 2. Locations of primers in cattle and buffalo species used for the mPCR (A and B).

post-PCR analysis; also, consume time or require skill for result interpretation thereby delaying the decision. While, the real-time PCR is highly sensitive, does not require post-PCR processing and has higher precision but requires costly reagents and equipment thereby precluding its application in routine laboratories.

Although, species-specific PCR can identify the target DNA accurately in a short period of time, but the mPCR has ability of simultaneous detection of more than one species at a time. In similar test, as many as six animal species were detected by mPCR in a single reaction tube making the technique a method of choice for animal species identification (Matsunaga *et al*., 1999). For this reason, multiplex PCR saves time, labor and preferred for mass screening of samples. However, multiplex PCR to detect and differentiate buffalo meat with beef are scarce; hence, this novel mPCR would act as tool for laboratories engaged in species identification.

In other similar studies, duplex PCR (De *et al*., 2011), real-time PCR (Dalmasso *et al*., 2011) and PCR-RFLP (El-Rady and Sayed, 2006) assays have been used for differentiation of cattle, buffalo and other species. Multiplex PCR assay developed in the present study was able to correctly identify cattle and buffalo DNA in a single reaction. In a similar study, goose and duck meat were differentiated using a multiplex PCR with a common forward and species-specific reverse primers to nuclear repeated target (5S rDNA gene) in order to detect the fraudulent substitution of a duck liver for the more costly goose liver (Rodriguez *et al*., 2001). Bataille *et al.* (1999) used cyt b gene and mitochondrial D-loop region to differentiate human and animals using a multiplex PCR where appearance of two bands was indicative of human and a single band in the animals. In a highly promising multiplex PCR, Matsunaga *et al.* (1999) successfully employed multiplex PCR for identification of six animal species (cattle, pig, chicken, sheep, goat, and horse) using the common forward and species-specific reverse primers; however, buffalo was not included in six species.

CONCLUSION

Authentic identification and differentiation of cattle and buffalo species is warranted due to adulteration, religious, social, forensic, and public health issues. In order to address these problems, a multiplex PCR was developed for the identification and differentiation of cattle and buffalo species using species-specific forward and common reverse primers flanking mitochondrial hyper-variable D-loop region. The mPCR amplicons of size 382 bp in cattle and 336 bp in buffalo were found highly diagnostic; difference of 46 bp between the PCR products of two species was adequate for the identification and differentiation of these species. In the milieu of social, religious, economic, forensic, and public health issues relating to cattle and buffalo species identification, novel mPCR developed in this study could benefit laboratories engaged in the identification and differentiation of cattle and buffalo species.

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