J.Meat Sci., 2016, 11 (2) : 70 - 74

Research Note

Species Authentication and Sex Differentiation of Cattle and Buffalo Meat using Polymerase Chain Reaction and Restriction Fragment Length Polymorphism of Amelogenin XY Gene

***P.S. Girish[1], S. Vathiyanathan, Shailesh Bagale[2] and Nagappa Karabasanavar[3]**

ICAR – National Research Centre on Meat, Chengicherla, Hyderabad, T.S
[1]ICAR – National Research Centre on Pig, Rani, Guwahati, Assam
[2]Department of Animal Husbandry& Veterinary Services, Belgaum, Karnataka
[3]Veterinary College, KVAFSU, Shimoga, Karnataka

ABSTRACT

Production, consumption and export of cattle meat (beef) are restricted, while that of buffalo meat is permitted without much restriction in India. Guidelines for prevention of cow slaughter are more stringent as compared to that of bullocks/ bulls. This necessitates authentic species and sex detection tools. In the present study, a technique based on polymerase chain reaction –restriction fragment length polymorphism (PCR–RFLP) has been developed for simultaneous detection of species and sex of cattle and buffalo meats. Amelogenin XY genes pecific PCR yielded two amplicons of size 280 and 217 bp in males and a single amplicon of 280 bp size in females. Further, restriction digestion of purified 217 bp Amelogenin Y amplicon yielded two fragments of size 133 bp and 84 bp in buffalo only (uncut in cattle). Results revealed that PCR–RFLP XY gene can be a convenient and quick assay for sex and species differentiation of beef and buffalo meat.

Keywords : *Beef, Buffalo meat, Speciation, Sexing, PCR, Amelogenin XY*

Received : 20.03.2016 Accepted: 28.05.2016

Slaughter of cattle is regulated in India; slaughter of both male and female cattle is prohibited in few states and slaughter of only female cattle are prohibited in most of the states. In addition, there is a complete ban on the export of cattle meat (beef). However, no restrictions have been imposed either on slaughter or sale/ export of buffalo meat. Species and sex authentication of meat is essential owing to religious, trade, legal and economic considerations. Since majority of female buffaloes are slaughtered after their productive life and males are slaughtered young in their life, the later provide superior quality meat. Sexing of meat determines consumer confidence and ensures label claims; hence, they require authentic sexing and speciation tools.

World wide, beef derived from male cattle is preferred over the females due to their better eating qualities requiring sexing of beef (Zeleny and Schimmel 2002). Therefore, cases of fraudulent adulteration of buffalo meat with beef often emerge requiring confirmation of meat and other forensic/legal samples for species and sex differentiation. In order to confirm the origin of species and also to differentiate the sex origin of meat there is a need for reliable laboratory tests. Although, several methods have been developed for this purpose, yet there is a need to develop a technique for the simultaneous identification of species and sex of cattle and buffalo meat.

Animal species could be authenticated using electrophoretic (Bhilegaonkar *et al*. 1990), isoelectric focusing (King 1984), counter-immuno-electrophoresis (Sherikar *et al*.1993), chemometric analysis (Ioannis and van Maria 2003), enzyme linked immune sorbentassay (Martin *et al*. 1991) or polymerase chain reaction (PCR) and its advanced variants (Girish *et al.* 2004; 2005; 2013). Likewise, methods have been developed for gender differentiation of meat samples using different techniques *viz*., H-Y antigen detection (Wachtel 1984), cytology (King 1984), enzyme linked immunosorbent assay (Simontacchi *et al*. 1999), High Pressure Liquid Chormatography–Mass Spectrophotometry/ Mass Spectrophotometry (HPLC–MS/MS) (Draisci *et al*. 2000) and Gas Chromatography (GC) –MS (Hartwig *et al*.1997). Several molecular techniques have also been developed for the identification of sex in animals. The DNA based PCR targets for sexing include SRY gene (Bai *et al.* 2010), zinc finger (ZFX and ZFY) genes (Kirkpatrick and Monson 1993; Zinovieva *et al.* 1995), amelogenin XY gene (Ennis and Gallager 1994), BRY gene(Matthews and Reed 1992) or single copy sequence BOV97 M (Miller and Koopman 1990).

The PCR amplification of Amelogenin (AMEL) gene which exists on both X (AMELX) andY (AMELY) chromosomes has been the promising target for animal sex identification.

*Corresponding author E-mail address: girishlpt@gmail.com

Amelogenin XY gene was targeted for sexing different meat species *viz*. cattle (Ennis and Gallager 1994), sheep/ deer (Pfeiffer and Brenig 2005; Gokulakrishnan *et al*. 2013) and otherspecies of the family *Bovidae* (Weikard *et al*. 2006). Molecular techniques are preferred over conventional methods for both species and sex differentiation of meat owing to their rapidity, sensitivity, applicability and cost advantages.

Objective of this study was to standardize a technique for simultaneous authentication of species and sex of cattle and buffalo meat by PCR (differentiation of sex) and RFLP (species authentication) technique targeting Amelogenin XY gene.

Sample collection and DNA extraction: Meat (100 g) samples of cattle (male) and buffalo (male and female) were collected from Municipal slaughterhouse, Chengicherla, Hyderabad and stored at 20°C until further analysis. Blood (5 ml in ethylene diamine tetra acetate) samples of female cattle (cow) were collected from Dairy farm of Veterinary College, Hyderabad. About 20 samples were collected from each category of meat sample. Extraction of DNA from tissue and blood samples was carried out as per the method described by Chikuni *et al*. (1994) and Sambrook and Russel (2001). Sample DNA showing OD 260/280 ratio between 1.76 and 1.89 was used as template for PCR amplification.

Amelogenin XY gene PCR: Amelogenin XY gene specific oligonucleotide primers of cattle and buffalo asreported by Ennis and Gallager (1994) *viz*., forward primer 5′ - CAG CCA AAC CTC CCTCTG C - 3′ and reverse primer 5′ - CCC GCT TGG TCT TGT CTG TTG C – 3′ were custom synthesized and used for PCR amplification (Bioserve Biotech Pvt. Ltd, Hyderabad). Amplification was carried out in 0.2 ml PCR tubes containing 2.5 μl of 10X PCR buffer(100 mMTris–HCl, pH 9.0, 15 mM $MgCl_2$, 500 mMKCl and 0.1% gelatin), 1 μl of 10 mMdNTP mix, 1 μl (20 pmol) each of forward and reverse primers, 1 IU of Taq DNApolymerase, 50 ng of purified DNA and autoclaved Milli- Q water to make volume up to 25 μl. Conditions on a Master Cycler gradient thermocycler (Eppendorf, Germany) optimizedwere: 5 min at 94°C for initial denaturation, followed by 34 cycles of amplification (45 S at 94°C, 45 S at 60°C and 1 min at 72°C) and final extension for 10 min at 72°C. The PCR products were analyzed by electrophoresis in 2% agarose gel with ethidium bromide staining.

Purification of amplicons: Amelogenin X and Y gene PCR products were cut from the agarose gel and purified by gel extraction kit (Bangalore Genei Pvt. Ltd., Bangalore, India). Purified amplicons were electrophoresed (2% agarose gel) to confirm the sizes of respective amplicons.

Restriction enzyme digestion of PCR products: Based on the restriction map of Amelogenin X and Y genes, *Alu*I restriction enzymewas found to differentiate cattle and buffalo species, hence was used to digest the resultant PCR products. Ten micro liters of PCR products were digested for 12 hours at 37° C using 5IU of *Alu*I restriction enzyme (M/s Bioserve Biotechnologies Pvt. Ltd., Hyderabad) in 10Xbuffer (25 μl reaction volume). Products of restriction digestion were separated by agarose (2%) gel electrophoresis and images were analyzed.

Sexing cattle and buffalo meat by Amelogenin XY gene PCR: Amelogenin XY gene PCR amplified two products of size 280 and 217 bp in males; however, a single PCR product of size 280 bp was amplified in females of cattle and buffalo species (Fig. A1 and Table 1). Amelogenin XY gene has been widely targeted for animal sexing, embryo or tissue samples of cattle (Ballin and Madsen 2007; Gokulakrishnan *et al*. 2012); sheep (Dervishi *et al*. 2008) and humans (Gibbon *et al*. 2009). Normally, males carry XY and females XX chromosomes; differences in the sequence of Amelogenin gene at X and Y chromosomes helps in the sexing of animals. Similar studies by other investigators alsoshowed two bands specific to male (♂) and a single female (♀) specific band *viz*., ♂263 +218 bp: ♀263 bp (Pfeiffer and Brenig 2005); ♂130+67 bp: ♀130 bp (Ballin and Madsen 2007); ♂189+126 bp: ♀189 bp (Gokulakrishnan *et al*. 2013). Amelogenin gene PCR has been found reliable for sexing domestic and wild species of bovines, ovines, caprines and most of the mammalian species (Iwase *et al*. 2003: Weikard *et al*. 2006). When compared to another sexing target SRY gene based PCR that indicates sex of animals based on presence/ absence of Y chromosome specific amplicons, targeting Amelogenin gene is beneficial due to the fact that the later rules out the chances of false negatives that could arise due to amplification failure. Bai *et al.* (2010) reported a duplex PCR targeting SRY and mitochondrial 12S rRNA genes to overcome the false negatives. Further, as Amelogenin PCR itself acts as an internal control the issues linked to false negative reaction could be overcome (Dervishi *et al*. 2008).

Speciation of cattle and buffalo meat using RFLP of Amelogenin gene PCR products: The *Alu*I restriction enzyme has a site to digest Amelogenin X gene amplicon of 280 bp PCR product into two fragments of size 260 and 20 bp in cattle but not in buffalo. However, due to smaller size of restriction fragment of 20 bp and very little difference between cut and uncut fragments (260 and 280 bp) it was found difficult to detect the difference in agarose gel electrophoresis (Fig. 1B) hence found to be less useful in routine analysis. However, RFLP with *Alu*

I restriction enzyme of Amelogenin Y gene was more convincing as compared to that of Amelogenin X gene. *Alu*I digestion of 217 bp PCR product of Amelogenin Y gene showed two fragments of size 133 and 84 bp in case of buffalo but not in cattle. Although, 84 bp fragment size after digestion was difficult to detect in agarose gel electrophoresis (Fig 1B), digested fragment of 134 bp was very easy to detect hence can be of use for differentiation of species in meat derived from male meat animals. Thus RFLP pattern of Amelogenin XY gene differentiated cattle and buffalo species. Experiment was repeated with about twenty samples originated from cattle and buffalo and the result was found to be repeatable.

Duplex PCR developed by Bai *et al*. (2010) targeting SRY and mitochondrial 12SrRNA genes overcomes false negative problem; however, it will not detect animalspecies while species identification techniques based on RFLP do not differentiate sex of meat (Girish *et al*. 2005). The Amelogenin Y gene based PCR-RFLP techniquedeveloped in this study could simultaneously identify the sex and species of origin in male cattle and buffalo meat samples. Due to restrictions on slaughter of cattle in different states of India the technique described in the present study would help the laboratories to undertake sexing and speciation of suspected meat samples which would help in solving issues linked to the meat adulteration, trade, legal, forensic and export of the beef.

The PCR - RFLP technique developed in the present study detects origin of species and sex of cattle and buffalo meats. In the first step, Amelogenin X and Y genes areamplified by PCR where female cattle and buffaloes show a single Amelogenin X genespecific amplicon of size 280 bp and males two amplicons of size 280 and 217 bp specific to Amelogenin X and Y genes. While RFLP of purified Amelogenin Y (217 bp) gene with *Alu*I restriction enzymeidentify species origin of meat derived from male animals. Restriction fragments of size133+84 bp after digestion of 217 bp Amelogenin gene confirms that meat is derived from buffalo species while undigested Amelogenin Y gene indicates that meat is from cattle. This approach could be used for solving issues linked to tradeand legal disputes requiring confirmation of species and sex of cattle and buffalo meat.

ACKNOWLEDGEMENTS

Authors would like to sincerely thank the Director, ICAR - National Research Centre on Meat, Hyderabad for providing financial assistance and laboratory facility to carry out this study

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Table 1: PCR and RFLP results for interpretation of species and sex in cattle and buffalo

				Digestion with AluI (RFLP) Species	
	Sex	**Bands**	**Size (bp)**	**Cattle**	**Buffalo**
Appearance	7.27 ± 0.12^{c}	6.47 ± 0.12^{b}	6.37 ± 0.13^{ab}	6.07 ± 0.14^{a}	
Amelogenin XY	Male	Two	280	260+20	280*
gene PCR			217	217*	133+84
	Female	One	280	260+20	280*

*Uncut

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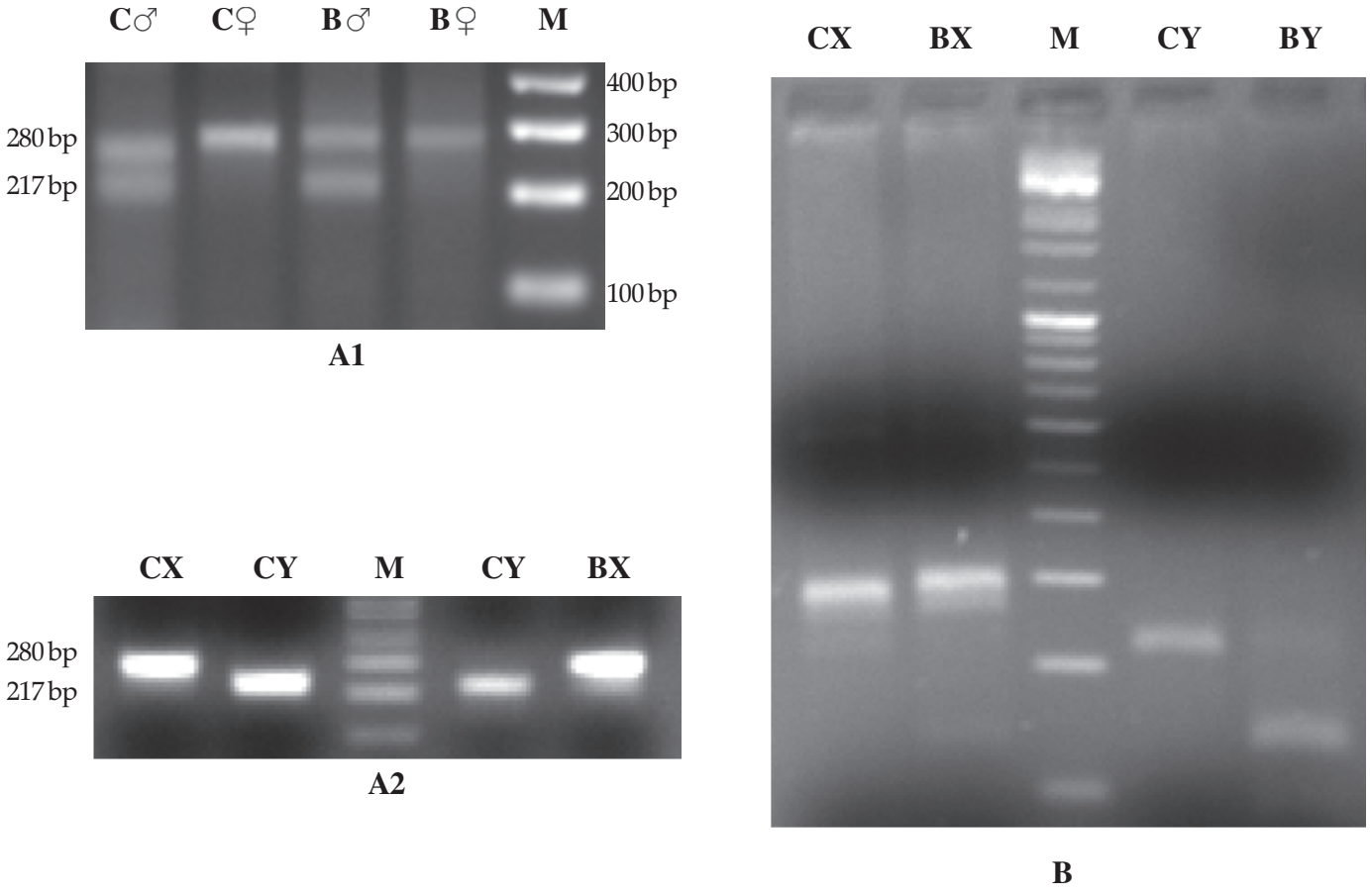

Fig. 1: Differentiation of sex in cattle or buffalo meat using PCR-RFLP of Amelogenin XY gene (A1: PCR products, A2: purified PCR products and B: RFLP using *Alu*I)

(C: cattle, B: buffalo, ♂: male, ♀: female, M: 100 bp DNA Ladder; X: Amelogenin X gene, Y: Amelogenin Y gene)