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Characterization of 5′ Upstream Region and Identification of Polymorphism in Intron 1 of Prolactin (*PRL*) Gene using *Hae*III PCR-RFLP in Indian Cattle Breeds

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ABSTRACT

Prolactin (*PRL*) gene is an important lactogenic candidate gene, plays a crucial role in mammary gland development and in the initiation, maintenance of lactation and expression of milk protein genes. In the present study, characterization of *PRL* 5′ upstream region and investigation of status of intron 1 polymorphism was carried out in Indian cattle breeds. An 857 bp fragment of 5′ upstream region of *PRL* gene consisting of part of promoter, exon1 and partial intron 1 was amplified by PCR and subsequently sequenced in Indian breed of cattle. Nucleotide sequences of *PRL* 5′ upstream region exhibited a high degree (>98%) identity among Indian as well as exotic cattle breeds. *Hae*III polymorphism screening in *PRL* intron 1 of Indian cattle breeds including Sahiwal (n = 154) and Hariana (n = 50) revealed monomorphic pattern, only, AA genotype (deletion homozygote) was found and confirmed by sequencing. The obtained sequences of *PRL* after aligning was revealed absence of *Hae*III recognition site GGCC due to deletion of G and consequently, we could not perform the association study of this deletion with milk production traits.

Keywords: Prolactin, Sahiwal, Hariana, Cattle, *Hae*III, PCR- RFLP, milk production traits

Prolactin (*PRL*) is a polypeptide hormone, secreted mainly by the anterior pituitary gland and involved in many reproductive pathways (Kruger *et al*., 2003). It is responsible not only for triggering lactation but also for mammary gland growth and lactogenesis (Collier *et al*., 1984). Bovine *PRL* gene is located on chromosome 23 and comprises five exons and four introns spanning a 10 kb genomic segment and encodes a 199 amino acid mature protein (Cao *et al*., 2002). The expression of *PRL* depends on the 5′ flanking region sequence. The 5′ flanking region of the *PRL* gene has been considered as an excellent experimental model for studying both tissue specific and hormonally regulated activation of gene transcription.

India has the largest cattle population which constitutes around 15.0% of world cattle population (FAOSTAT, 2015). It also has the distinction of possessing 40 well descriptive breeds (NBAGR, 2016). Therefore, the bovine *PRL* gene seems to be an excellent candidate for linkage analysis with quantitative trait loci (QTL) affecting milk production traits (Brym and Kaminski, 2006). Characterization of the *PRL* regulatory region of different cattle breeds of India may lead to the identification of single nucleotide polymorphisms (SNPs) which can be used as potential marker for selection to improve the milk production (Mahmoud and Nawito, 2012).

Several polymorphic sites have been detected within *PRL* gene and statistically significant associations between *PRL* variants and milk production traits have been described in dairy cattle (Dybus *et al*., 2005; Ghasemi *et al*., 2009). Polymorphism study in *PRL* 5′ upstream region has also been reported in non-Indian cattle (Uddin *et al*., 2013) and buffalo (Ladani *et al*., 2003; Madnalwar *et al*., 2009).

Considering lack of such information in Indian cattle breeds, the present study was undertaken to characterize the *PRL* 5′ upstream region and investigate the status of *Hae*III polymorphism in *PRL* intron 1 of Sahiwal and Hariana cattle breed.

MATERIALS AND METHODS

Animals and DNA Isolation

For PCR-RFLP study, blood samples were collected randomly from a total of 154 animals of Sahiwal cattle breed maintained in NDRI, Karnal (n=102) and Instructional Livestock Farm Complex (ILFC), DUVASU, Mathura (n=52) and 50 Hariana cattle maintained at ILFC, DUVASU, Mathura. Genomic DNA was isolated using the standard phenol–chloroform extraction method (Sambrook and Russel, 1991). The concentration and purity of genomic DNA was determined spectrophotometrically at OD_{260} and OD_{280}. The integrity of the DNA was examined by agarose gel (0.7%) electrophoresis and visualized the gel under UV light after staining with ethidium bromide (EtBr).

Amplification of PRL upstream region

An amplicon of 857 bp consisting of *PRL* promoter region (235 bp), exon I (83 bp) and intron I (539 bp) was amplified using a specific primer pairs (PRL/start F: 5′-ATTATCTCTCTCATTTCCTTTCA-3′ and PRL/stopR: 5′- ACTCTGCTGTCACTGTCTGTATT -3′; Zhang *et al*., 1994). PCR amplification was carried out in a total volume of 25 µl that contained 1.0 µl of genomic DNA (50-100 nmoles), 1X PCR buffer (10 mM Tris–HCl, pH 8.8 at 25°C, 50 mM KCl), 2.5 mM of $MgCl_2$, 2.5 mM of each dNTPs, 4 pmoles of each primer and one unit of *Taq* DNA polymerase (Fermentas, USA). The cycle conditions included an initial period of denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 50 sec and extension at 72°C for 50 sec, and a final extension at 72°C for 10 min. The PCR product was checked by agarose gel (1.0%) electrophoresis in 1X TAE buffer after staining with EtBr.

Sequencing and Sequence analysis

The positive amplified products were sequenced commercially by automated sequencer using standard cycle conditions by Sanger′s dideoxy chain termination method. The sequences obtained were subjected to BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) to ascertain whether the obtained sequences corresponded to *PRL*. The nucleotide sequences of *PRL* 5′ upstream region of Sahiwal and Hariana breeds were aligned with those of other exotic cattle breeds and species available in the GenBank database using the Clustal method of MegAlign programme of Lasergene software (DNASTAR, USA) and BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). A phylogenetic and molecular evolutionary analysis was conducted using MEGA version 4.0 (Tamura *et al.*, 2007).

In addition to the *PRL* 5′ upstream sequences characterized in the present study, sequences available in GenBank such as exotic cattle (AF426315; X01452), yak (AF516697), and goat (EU586510) were also used for sequence analysis.

Genotyping of animals by *HaeIII* PCR-RFLP assay

The amplicon of 857 bp as described earlier was digested using restriction enzyme (RE) *Hae*III (Fermentas, USA). The restriction digestion was carried out at 37°C for 14 hr in a total volume of 15µl containing 5µl PCR products, 1.0 µl *Hae*III (10U/µl) and 1.5µl 10X RE buffer. For restriction fragment analysis, digested products were checked on 1.5% agarose gel in 1X TAE buffer for 4-5 hrs at 5 V/cm. The fragments were visualized under UV light after staining with EtBr. Restriction analysis indicated that the wild-type homozygote (G/G) possessing a polymorphic *Hae*III site generated 2 fragments (662 and 195 bp) and was referred as BB. Due to the absence of polymorphic *Hae*III site, the mutant homozygote (-/-) yielded uncut fragments (857 bp) and was named as genotype AA. The data was generated by estimating the frequency of different RFLP pattern. The allelic frequency and genotypic frequencies of *PRL* gene was estimated by standard procedure (Falconer and Mackay, 1996).

RESULTS AND DISCUSSION

PRL genomic fragment of 857 bp has been amplified and sequenced in Sahiwal (GenBank Acc. no. KM242132) and Hariana (KP635967) breeds of Indian cattle. Comparative analysis of 857 bp *PRL* 5′ upstream region between Sahiwal and Hariana cattle breeds revealed 99.1% identity.

Table 1: Percent identity of *PRL* gene of different Indian and exotic breeds of cattle with other related species on the basis of nucleotide sequences using DNASTAR software

Percent Identity									
1	**2**	**3**	**4**	**5**	**6**		**Breed/Species**	**Acc. No.**	
1		98.2	97.4	94.0	98.6	97.9	**1**	*Bos gruiennens*	AF665197
2	1.1		99.3	99.5	99.4	98.7	**2**	Bos taurus	AF426315
3	0.6	0.5		93.7	98.6	97.9	**3**	*Bos tauras*	X01452
4	4.5	4.7	4.4		94.9	94.3	**4**	Capra hircus	EU586510
5	0.7	0.6	0.1	4.5		99.1	**5**	Hariana Cattle	KP635967
6	0.7	0.6	0.1	4.5	0.2		**6**	Sahiwal cattle	KM242132
	1	**2**	**3**	**4**	**5**	**6**			

They showed 98.1% - 99.4% identity with exotic cattle breed, while 94.3% – 94.9% and 97.9% - 98.6% sequence identity with goat and yak, respectively (Table 1). Phylogram (Fig. 2) based on nucleotide sequences of *PRL* 5′ upstream region revealed that the *Bos* genus including cattle and yak formed a separate cluster away from the *Capra hircus*. Within *Bos* genus, Indian cattle breeds (*Bos indicus*) including Hariana and Sahiwal formed a separate branch leaving *Bos tauraus* and *Bos grunniens* sequences.

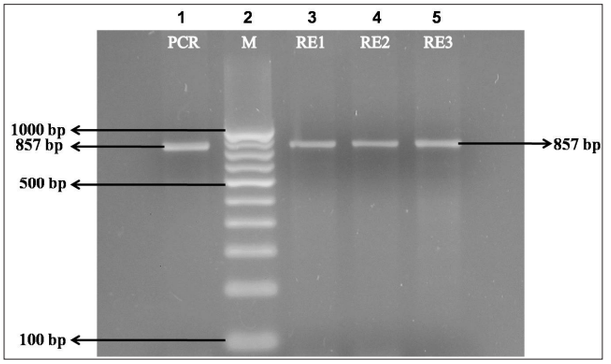

Fig. 1: PRL/*Hae*III PCR-RFLP assay pattern in 1.5% agarose gel; Lane 1: Undigested PCR product (857 bp), 2: Marker (StepUp 100 bp DNA ladder, Cat No. MBD13), 3-5: AA genotype (uncut band; 857 bp)

The lack of variation in the *PRL* 5′ upstream region might be due to the fact that presence of binding sites for sugars, proteins and other molecules at this site of the DNA chain that possibly influence the process of DNA transcription. These regions are characterized by low mutation rates during evolution due to their importance for the maintenance of protein synthesis. This high conservation may be significant by indicating that the regulation of this gene is under extremely rigid control by transcription factors, with high conservation being necessary for full activation.

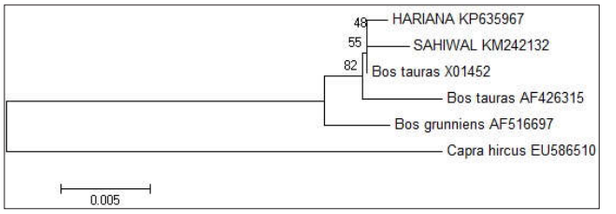

Fig. 2: Phylogram based on nucleotide sequences of different cattle breeds along with different species *PRL* 5′ upstream region using the boot strap/Neighbor-Joining tree method using MEGA4. The percentage of replicate trees (boot strap value) in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

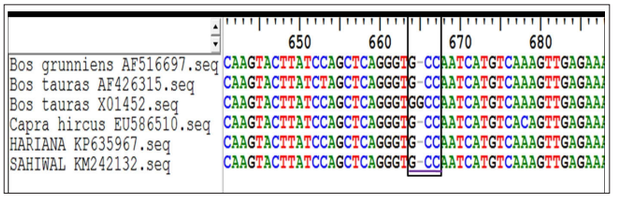

Fig. 3: PRL/*Hae*III DNA Sequencing showing deletion of G at GGCC recognition site of *Hae*III restriction enzyme. Recognition sequence of *Hae*III is underlined

Restriction digestion and sequence analysis indicated the presence of one polymorphic *Hae*III recognition site (GGCC) in the amplicon at 663 bp downstream to forward primer (Fig. 3). PRL/*Hae*III assay revealed that all the

screened Sahiwal and Hariana cattle were homozygous for allele A and we could not identify any animal with wild type homozygote (G/G) and heterozygote genotype (G/-). This suggests that a restriction site might be abolished due to deletion of G from recognition site of the *PRL* intron 1 region. The fixation of favourable allele A due to significantly high milk producing nature of Sahiwal cattle. Consequently, we could not establish any association of the observed polymorphism with milk production traits.

This finding was similar to the observations of earlier reports in cattle and buffalo by Zhang *et al*. (1994) in Holstein Friesian, Jersey and Hereford bulls, Ladani *et al*. (2003) in Surti and Mehsani buffaloes, Laureano *et al*. (2009) in Nelore cattle and Madnalwar *et al*. (2010) in Pandharpuri buffalo.

CONCLUSION

In the present study we report the characterization of the *PRL* 5′ upstream region and also identified the G deletion in intron 1 region but we could not establish any association between genotype and milk production trait because these cattle were found homozygous for this deletion. It would be interesting to further investigate the roles of these putative SNPs and deletion identified in this study for better perceptive of the transcriptional regulation.

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