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SEQUENCE CHARACTERIZATION OF LACTOFERRIN GENE PROMOTER REGION IN *BOS INDICUS* CATTLE

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ABSTRACT

Prevalence of several diseases especially mastitis in dairy animals causes reduction in milk production leading to heavy economic loss to the dairy farmers. Lactoferrin (Lf) is a minor whey non-heme iron binding protein produced by the mammary gland may serve a dual role, protecting both the mammary gland and the neonatal intestine from infection. The characterization of lactoferrin gene promoter region has been described in *Bos taurus* cattle, but there are no reports available in *Bos indicus* cattle which are widely distributed in Indian sub-continent. About 1kb sequence of lactoferrin gene was generated and the BLAST analysis revealed 99% sequence homology with the exotic cattle sequence. The phylogenetic analysis showed clustering of indicus and taurine cattle together in a single node indicating 5' regulatory region of lactoferrin gene is highly conserved in these two sub-species. A total of 13 putative transcription factor binding sites were indentified. Although it seems unlikely that all the putative transcription factors identified in the promoter region exert their individual effects, some may actually influence the regulation of bovine lactoferrin gene expression in various tissues especially in mammary gland which need to be further assessed for their functional significance.

Key words: Lactoferrin gene promoter mastitis, Sahiwal.

INTRODUCTION

India ranks first in milk production (Anonymous, (112.5 MT), 2010) and possesses world's highest cattle population. However, productivity is low due to vast number of local non-descript animals, occurrence of diseases, inadequate availability of feed and fodder and under exploitation of genetic potential.

Prevalence of several diseases especially mastitis in dairy animals causes reduction in milk production. It reduces not only the milk yield but also changes the milk composition leading to heavy economic loss to the dairy farmers. Lactoferrin (Lf) is a minor whey non-heme iron binding protein with molecular weight of 80 k Da containing 708 amino acids, first identified in milk whey and also found present in most exocrine fluids, such as saliva, bile, pancreatic fluid, tears, bronchial and nasal secretions. The biological functions of lactoferrin include protection against pathogenic microbial infection, enhanced intestinal iron absorption in infants, promotion of cell growth and modulation of inflammatory response (Brock, 2002). Lf produced by the mammary gland may serve a dual role, protecting both the mammary gland and the neonatal intestine from infection. The mean concentration of Lf in the milk of cows with sub-clinical mastitis (0.2-1.2 mg/ml) has been shown to be higher in milk than that of normal cows (Hagiwara *et al.*, 2003). In addition, Lf concentration in sub-clinical mastitis might depend on the pathogenicity of each bacterial species; the mean Lf concentrations in milk from quarters infected with *S. aureus* or streptococci were significantly higher than in milk from quarters infected with coagulase-negative staphylococci (CNS) and *Corynebacterium bovis* (Hagiwara *et al.*, 2003). In cows with clinical mastitis, Lf concentrations in milk can range from 0.3 to 2.3 mg/ml; these concentrations are generally higher than in normal cows or those with sub-clinical mastitis (Kawai *et al.*, 1999 and

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Hagiwara *et al.,* 2003). The characterization of lactoferrin gene promoter region has been described in Holstein-Friesian cattle (Seyfert *et al.*, 1994 and Li *et al.,* 2004), but there are no reports available in *Bos indicus* cattle. Therefore, the present study was carried out with the objective to sequence characterize the bovine lactoferrin gene promoter region in Sahiwal breed of *Bos indicus* cattle.

MATERIALS AND METHODS

This is the first attempt to sequence characterize lactoferrin promoter region in Indian zebu cattle. The experimental animals utilized for the present study were from the herd of Sahiwal cattle maintained at cattle yard of National Dairy Research Institute, Karnal. Ten ml blood was collected aseptically by jugular vein puncture in a sterile vacutainer containing 15% of 0.12 ml EDTA solution (Beckton-Dickinson vacutainer). The samples were transported to the laboratory in an icebox and stored at 4 ^{0}C till further processing for DNA isolation. DNA was isolated from 10 ml of blood by phenol-chloroform method, as described by Sambrook *et al.* (1989) with few modifications. Quality and quantity of DNA isolated was estimated by spectrophotometer method. The promoter region (1122bp) of lactoferrin gene was segmented into five fragments (Lf 5'-I, Lf 5'-II, Lf 5'-III, Lf 5'-IV and Lf 5'-V) and five sets of reported primers of *Bos taurus* (Li *et al.,* 2004) (Table 1) were used to amplify these segments. Amplified PCR products one from each of five fragments of promoter was subjected to custom DNA sequencing from both ends (5' and 3' ends). Sequence data was analyzed using Chromas (Ver. 1.45, http://www.technelysium.com.au/chromas.html). Multiple sequence alignments were performed with Megalign program of LASERGENE software (DNASTAR, Inc, Madison WI, U.S.A). The 5'flanking region of lactoferrin gene was subjected to basic local alignment search (BLAST) to know the sequence homology with the corresponding regions of other species. Phylogenetic tree was constructed following the Neighbour Joining (NJ) and Unweighted Paired Group Method with Arithmetic Mean (UPGMA) procedures using CLC Free Work Bench software available online (www.clcbio.com).

TABLE 1: Primer sequence of different promoter segments of lactoferrin gene.

Promoter / Exon	Size		Primer Sequence
Fragment I	1122	FP:	5'-gtc tga acc tac aca tgc tg-3'
		RP:	5'-tcc tca gta cac agg ctg ac-3'
Fragment II		FP:	5'-gtt cct gtc tcc cac ctc ata-3'
		RP:	5'-ggg aac cag tta aga cag acg -3'
Fragment III		FP:	5' –gca gcg ggc cct ctt tca-3'
		RP:	5'- tgc tct ttc ttt ccc act tgt cc- 3'
Fragment IV		FP:	5'-gct gat ccg caa aga ttc ac-3'
		RP:	5'-gac cga ggg agc gag aaa g-3'
Fragment V		FP:	5'-ttt ctc gct ccc tcg gtc t-3'
		RP:	5'-ccc cgc ccc cac tca tac-3'

A search for putative transcription factor binding sites was performed using TESS (Schug and Overton, 1997) and MATCH (Kel *et al.,* 2003). The TRANSFAC database available on the public domain was utilized to search for the presence of different motifs in the 5'flanking region of lactoferrin gene. The algorithm of MATCH uses two score values; the matrix similarity score and the core similarity score. These two scores measure the quality of the match between the sequence and the matrix, which ranges from 0 to 1, where 1 denotes an exact match. The core of each matrix is defined as the first five most conserved consecutive positions of a matrix as described by Kel *et al.* (2003).

RESULTS AND DISCUSSIONS

A total of 1041 bp sequence of 5' flanking region of *Bos indicus* (Sahiwal) lactoferrin gene including partial exon 1 was generated in the present study (Accession no. EU170158.1). BLAST analysis of 1.041 kb (promoter) 5' flanking region was performed to find the sequence homology between various species. The BLAST results revealed 99%, 95%, 91%, and 93% sequence homology with *Bos taurus, Bubalus bubalis, Capra hircus* and *Ovis aries* respectively (Table 2). Accession number of the species, query coverage and percent homology to which BLAST analysis performed are presented in Table 2. The phylogenetic tree constructed based on neighbour joining (Fig.1) method (330 bp region) revealed clustering of cattle species *Bos taurus* and *Bos indicus* in a single node, *Bubalus bubalis* and *B. depressicornis* and sheep (*Ovis aries*) and goat (*Capra hircus*) formed different groups respectively, while a phylogenetic tree constructed (775 bp region) based on Unweighted Paired Group with Arithmatic Mean (UPGMA) revealed clustering of cattle species (*Bos taurus* and *Bos indicus*) in a single node with high bootstrap value and *Bubalus bubalis* and *Capra hircus* in different nodes (Figure 2). This indicates

TABLE 2: Lactoferrin promoter sahiwal (*Bos indicus)* percent homology with other species.

Accession No.	Species	Query coverage	Homology
AY319306.2	*Bos taurus*	100%	99%
EF650854.1	*Bubalus bubalis*	97%	95%
AJ784283.1	*Capra hircus*	98%	91%
AF194987.1	*Bos grunniens*	74%	98%
AY689192.1	*Bos javanicus*	31%	99%
AF281090.1	*Bos sauveli*	31%	99%
AY689191.1	*Bos frontalis*	31%	99%
AF091638.1	*Bos grunniens*	31%	99%
AF091640.1	*Syncerus caffer*	31%	97%
AF091641.1	*Bubalus depressicornis*	31%	97%
AF091651.1	*Ovis aries*	28%	93%

the promoter region of lactoferrin gene is highly conserved between taurine and indicus cattle.

Putative transcription factors binding sites (TFBS) were identified using MATCH (Kel *et al.*, 2003) and Transcription Element Search Software (TESS). A total of 13 TFBS were identified in 1.041 kb 5' flanking region of Sahiwal cattle (Table 3) like C/EBP alpha (-904 to -916), CCAAT box (-665 to -676), HNF-4 (-663 to -653), ER (-29 to -47), MyoD (-9 to -21) ROR alpha 1 (-775 to -787 and -39 to -51); four Sp1 sites at -554 to -559, -190 to -199, -232 to -237 and -58 to -65; two TFIID sites at positions -493 to -498 and -81 to -88. Transcription initiation sites like CAAT signal at position -908 to -912, CAAT complement at -709 to -713, two GC signal at -191 to -196; -58 to -63 and a TATA signal from -21 to -27 were identified at 5' flanking region of Sahiwal cattle. The 5' untranslated region (5'UTR) found to be 39 bases length and the exon 1 was identified to be 82 bases length (Fig. 3). All the identified sites in the present study were having 100% core similarity match (core similarity score= 1) and more than 90% matrix similarity match (matrix similarity score= 0.9). Similar study (Daly *et al.*, 2006) was reported in Montebeliard and Normande cattle breeds in 5' flanking region of lactoferrin gene. In another study (Bahar *et al.*, 2011) on promoter region of taurine cattle indicates that cows with the BtLTF_H1a haplotype had increased lactoferrin protein concentration in milk at various time points over the lactation curves, compared to herdmates with the BtLTF_H2a haplotype. The G to A polymorphism at -190, located in a putative selectivepromoter factor1 (SP-1) binding site, was associated with a longer calving interval and decreased functional survival in Holstein-Friesian (Halloran *et al.*, 2010).

TABLE 3: Putative transcription factor binding sites in 5' flanking region of lactoferrin gene in Sahiwal.

FactorName	Position	Strand	CoreMatch	MatrixMatch	Length	Sequence
C/EBP Alpha	-904 to -916	+	1.000	0.892	13	gctTGCAAtcagt
ROR alpha1	-775 to -787	+	1.000	0.901	13	gaaacagGGTCAg
CCAAT box	-665 to -676	-	1.000	0.932	12	tcATTGGcaaat
HNF-4	-633 to -653	-	1.000	0.835	21	agacagcCTTTGggcactt
ROR alpha1	-39 to -51	+	1.000	0.915	12	ctcgtgaGGTCAc
ER	-29 to -47	-	1.000	0.954	18	tgaGGTCAccgagcactgg
MyoD	-9 to -21	+	1.000	0.933	12	gcgCAGGTggca
Sp1	390 (-554 to -559)	-	-	-	6	GGGCAG
Sp1	750 (-190 to -199)	-	-	-	10	GCCCCGCCCG
TFIID	451 (-493 to -498)	-	-	-	6	TTCAAA
Sp1	712 (-232 to -237)	-	-	-	6	GGTGGG
TFIID	861 (-81 to -88)	-	-	-	8	GGCTGGGG
Sp1	884 (-58 to -65)	-	-	-	7	AGGGCGG

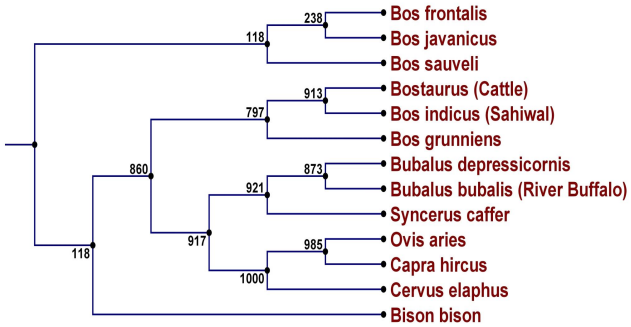

FIG. 1: Phylogenetic Tree of Partial 5' Flanking Region of Lactoferrin Gene (NJ Method) (330 bp Region).

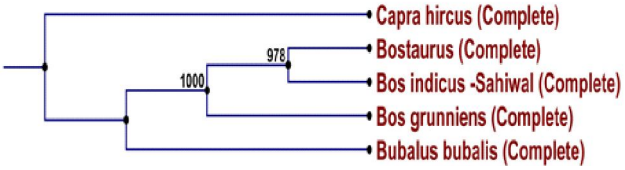

FIG. 2: Phylogenetic Tree of Partial 5' Flanking Region of Lactoferrin Gene (UPGMA Method) (775 bp Region)

Although it seems unlikely that all the putative transcription factors identified in the promoter region exert their individual effects, some may actually influence the regulation of bovine lactoferrin gene expression in various tissues especially in mammary gland. Hence, the 5' flanking region (promoter) of *Bos indicus* lactoferrin gene needs to be further assessed for its functional significance especially for their ability to bind transcription factors and alter gene expression.

FIG. 3: Lactoferrin 5' Flanking (Promoter) Region of Sahiwal showing putative transcription factor sites (Bold font).

CAAT signal

-948 TCCTCCCCCACCCCTTGGGGGACACTTAGTTTGCTTGCAATCAGTGAACGATAAGCAGG 59

-889 GCTGCACTGGAGACCCCTGCGTGGGAGTTGTTGTGCTTCAAGGGAGTGTCCTTCAAGGAT 119

-829 GCAGAGCAGAGTTCTAGCTTTAGAACTGAAAACCAGCCTCCTGAAACAGGGTCAGCCTGT 179

CAAT Complement

-769 GTACTGAGGACAAAATAGGACATTTATCAAAATGAGGTTCCTGTCTCCCTCCTCATATTG 239

-709 CCACAAAACAACACAAGGGGTAGGATATCCTTTTCATTGGCAAATGAGGGACCAGGAGAC 299

-649 AGCCTTTGGGCACTTAGGCCTCTGGTTCTGTTTTCTGGGAGCTGTATTACGGTCTCAGGA 359

Sp1

-589 GGACCCCAGGGGCAGTCTGGGTCAGACTCTGGGCAGCCTCTGCCAGCTGGACCAGGCTGC 419

TF II D

-529 CGTGGACCCCGGGCCAGGCAGCGGGCCCTCTTTCAAAACTCCAGGCTGGCTCTGCGTGCA 479

-469 GATGCAAGGGTCTCCGTCTGTCTTAACTGGTTCCCAAGCACTTTAGATACCTTCTCTATA 539

-409 GTCAAGCTGATCCGCAAAGATTCACCCTAGGACCCCTGCTCTGGATCCCGCTCTCTAGGA 599

-349 GGCACTGAGACCGGAGCGGGGACAAAACCCAGGGACTGCCACTCCCGAAGGGCTGCGGAC 659

Sp1

-289 AAGTGGGAAAGAAAGAGCATCCCCCAACTAGGCAGCGCTGGGGAACTTGAGAGGTGGTG 719

Sp1 GC Signal

-229 TGGGTTGGGTATCCTCTCCCCGAGCGCCAAGCCCCGCCCGGGCACCTTTCTCGCTCCCTC 779

-169 GGTCTCCACCCCCGCTCTTCCCCCTTCCCCCCGGTTTTCCCCCTCTAGGAACCAGCAGAC 839

TF II D Sp1 GC Signal

-109 CTCGGGAGAGGGGAGGAGGGAGGCTGGGGCGCTTATAGGACCACAGGGCGGGGCAAACCT 899

TATA signal EXON 1

-49 CGTGAGGTCACCGAGCACTGGCTAAAGGGACGCAGAACGAGCGCAGGTGGCAGAGCCTTC 959

5' UTR

12 GTTCCGGAGTCGCCCCAGGACGCCAGCCATGAAGCTCTTCGTCCCCGCCCTGCTGTCCCT 1019

72 CGGAGCCCTTGGTGAGTGCAGG---------- 1041

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