



## Single strand conformation polymorphisms (SSCPs) in the 5'-flanking region of Lactoferrin gene and its association with milk production traits and somatic cell score in Holstein cattle

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### ABSTRACT

Lactoferrin (*Lf*) is a bioactive protein present in all external secretions such as milk that plays important role in the innate host defense. Therefore, the *Lf* has a major role in bovine mammary gland defense during mastitis. In this study, 5'-flanking region of bovine *Lf* gene containing three single nucleotide polymorphisms (SNPs) at positions -602 (T/G), -600 (A/G) and -586 (T/C) were screened using PCR-SSCP method. Three distinct SSCP patterns, representing 3 different haplotypes (A, B and C), were identified. The frequencies of A, B, and C haplotypes for amplified fragment were 0.43, 0.39 and 0.18, respectively. An association study was applied with data from milk production traits and somatic cell score (SCS). Statistically significant differences were found between various haplotypes at the amplified fragment and fat percentage ( $P < 0.05$ ). However, the haplotypes also tend to associate with protein percentage ( $P = 0.06$ ) and milk yield ( $P = 0.08$ ). No significant difference was observed between combined genotypes and SCS. Therefore, the haplotypes identified in the 5'-flanking region of bovine *Lf* gene can serve as potential candidate genetic markers for marker assisted selection in cattle.

**Keywords:** 5'-flanking region, *Lf* gene, haplotype, milk production traits, SCS

### INTRODUCTION

Lactoferrin (*Lf*) is a cationic iron-binding glycoprotein found in the biological fluids of a wide range of mammalian species, such as bovine, human, goat and porcine. The sequence homology of the lactoferrin amino acids are highly conserved among the species of higher mammals (Teng, 2002). The primary function of lactoferrin is the defense against microbial infection, mainly through its strong ability to bind ferric ions required for microbial growth, direct interaction with bacterial surface (Legrand et al., 2008) and direct bactericidal activity due to lactoferricins (Bellamy et al., 1992; Van der Karaan., 2006). The *Lf* may also play a role in transcriptional activation (He and Furmanski, 1995) and suppression of tumor growth (Bezault et al., 1994).

The lactoferrin level of milk varies terrific between species, however, bovine milk contains about one-tenth of the lactoferrin found in other mammals with concentrations ranging between 0.02 to 0.2 mg/ml (Schanbacher et al., 1993). In dairy cattle, its concentration dramatically increases during the dry period and a mastitis infection (Kutila et al., 2003), suggesting that this protein plays important physiological roles in the reduction of mastitis incidence (Hagiwara et al., 2003).

A number of mastitis detection methods have been developed previously with varying degrees of accuracy and automation (De Mol et al., 1999; De Mol and Ouweltjes, 2001; De Mol and Woldt, 2001). Somatic cell (mostly cells of immune system) count (SCC) in milk presents a fast and reliable analytical tool. It is related to the immunological status of the udder and increases in response to an inflammatory stimulus like bacterial infection (O'Brien et al., 1999; Leitner et al., 2000). Many studies confirmed a positive and high correlation between mastitis and SCC, and therefore is a valuable component of monitoring programs (Schukken et al., 2003).

The gene coding for bovine *Lf* is located on chromosome 22 and contains 17 exons spanning about 34.5 kb of genomic DNA. The 5'-regulatory region of the bovine lactoferrin gene contains the promoter sequence, a non-canonical TATA box and a number of transcription factor binding sites necessary for expression of the gene (Zheng et al., 2005). There is a strong suggestion that polymorphisms which are located in a 5'-regulatory region have an impact on a gene expression (Shi and Teng, 1996; Wang et al., 1998; Stokes et al., 2004). Several genetic polymorphisms have been identified in the 5'-regulatory region of bovine *Lf* gene in various cattle breeds that few of them had significant effect on performance traits in dairy cattle (Kaminski et al., 2006; Huang et al., 2009; O'Halloran et al., 2009). Therefore, the objective of this study was to determine the haplotype frequency of polymorphisms at positions -602 (T>G), -600 (A>G), and -586 (T>C) of the 5'-flanking region of bovine *Lf* gene in Holstein cattle at Esfahan province and to evaluate their association with milk production traits and SCC.

### MATERIALS AND METHODS

Blood samples were collected in individuals from 600 Holstein cows in single farm at Isfahan province. AccuPrep® genomic DNA extraction kit was used to isolate genomic DNA from EDTA-anticoagulated peripheral blood samples.

Using Primer3 software (<http://frodo.wi.mit.edu/primer3/>), one new primer pair was designed from *Lf* sequence (GenBank accession no.AH000852). A 379 bp (-859 to -480 relative to transcription start codon site) fragment was amplified by polymerase chain reaction (PCR) using forward (5'-TGTTGTGCTTCAAGGGAGTG- 3') and reverse (5'-CCAGCCTGGAGTTTTGAAAG- 3') primers. Twenty five µl of polymerase chain reaction (PCR) mixture were carried out in 0.2 ml PCR tubes, using a PCR kit with the lyophilized components. Each tube contained 1.5 units of *Taq* DNA polymerase, 10 mM of Tris-HCl, 50 mM of KCl, 1.5 mM of MgCl<sub>2</sub>, 200 mM of each dNTP, 20 pmol of each primer and 50 ng genomic DNA. The following PCR conditions were used: denaturation at 94°C for 2 min; 30 amplification cycles of denaturation at 94°C for 45 s, annealing at 63°C for 45 s, extension at 72°C for 30 s and a final 7 min extension at 72°C.

For single-strand conformation polymorphism (SSCP) analysis 2 µL of the PCR product was mixed with 6 µl stop solution (95% formamide, 20 mM EDTA, 0.025% xylene cyanol and 0.025% bromophenol blue). The samples were heated for 5 min at 95°C and immediately cooled on ice. The total volume was loaded onto an 8% polyacrylamide gel (37.5:1 acrylamide/bisacrylamide). Electrophoresis was carried out at room temperature in 0.5×TBE buffer for 24 h. The gels were subsequently fixed in 10% ethanol, stained with 0.15% AgNO<sub>3</sub> and revealed with 1.5% Na<sub>2</sub>CO<sub>3</sub>.

### Statistical Analysis

The frequencies of SSCP patterns for amplified fragment in analyzed population obtained by directed counting. The associations between three haplotypes of the *Lf* regulatory region with SCC and milk yield traits were analyzed using the mixed procedure of SAS (SAS Institute Inc, Cary, NC, USA) according to the following linear model.

$$y_{ijklm} = \mu + G_i + S_j + L_k + Si_l + Di_m + e_{ijklm}$$

Where:

$y_{ijklm}$  : Phenotypic value of the milk production traits and SCC of the animal  $k$  with a genotype  $i$ ,  $\mu$ : overall mean,  $G_i$ : Fixed effect of the  $Lf$  haplotypes,  $S_j$  : fixed effect of season of calving,  $L_k$  : fixed effect of lactation,  $Si_l$  : random effect of sire,  $Di_m$  : random effect of dam and  $e_{ijklm}$  : random error

Traits analyzed with this model were average of daily milk yield, fat percentage, protein percentage and SCC. The distribution of SCC values was skewed. Thus the values of SCC were log transformed and converted into SCS (Shook, 1993).

## RESULTS

Three haplotypes (A, B, and C) were identified with one major haplotype predominating in the amplified fragment of bovine  $Lf$  gene. The major haplotype (A) occurred with a frequency of 43% genomic samples while the B and C haplotypes appeared with 39 and 18 % of population study, respectively (Figure 1).

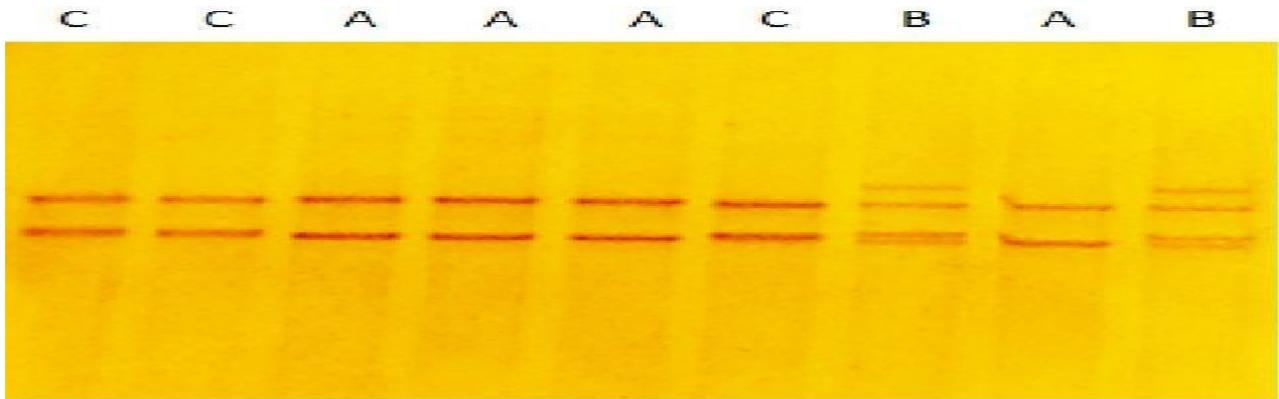


Fig.1: Different SSCP patterns of amplified fragment at the 5'-flanking region of bovine  $Lf$  gene.

The relationship between haplotypes with milk yield traits and SCS were evaluated. Results indicated that different haplotypes in this fragment had a significant association with fat percentage ( $P<0.05$ ). Cows expressing A haplotype had significantly increased fat percentage relative to the B and C haplotypes (table 1).

The combined genotypes of amplified fragment revealed to tend significant differences ( $P=0.08$ ) between observed genotypes and average daily milk yield. Holstein cattle expressing B haplotype had more milk yield. The identified haplotypes showed a high tendency to associate with protein percentage ( $P=0.06$ ) which cows with A genotype had more protein percentage vs. B and C genotypes. Association analysis failed to reveal any significant difference between detected haplotypes and SCS, but the C haplotype had more SCS rather than A and B haplotypes (Table 1).

Table1. Estimates of effects associated with different haplotypes in the 5'-flanking of  $Lf$  gene on average milk yield, fat percentage, protein percentage and SCS.

Variable	N	Mean	SD	P value	LS means for different haplotypes			P for contrast		
					A	B	C	A vs. B	A vs. C	B vs. C
Milk yield	591	33.07	6.27	0.08	35.27	37.85	35.73	0.02*	0.74	0.23
Fat %	586	3.35	0.52	0.02*	3.11	2.92	2.88	0.04*	0.05*	0.80
Protein %	577	2.94	0.44	0.06	2.77	2.62	2.59	0.09	0.08	0.76
SCS	591	5.44	1.03	0.27	6.70	6.57	6.97	0.46	0.18	0.11

## DISCUSSION

The 5'-flanking region of functional genes contains the elements that regulate the transcription process which may keep functionally relevant polymorphisms with major effects on gene expression. To date, genetic variations in the 5'-flanking region of bovine *Lf* gene has been extremely described. Hence, the present study was conducted to examine the association between genetic variation in the 5'-flanking region of *Lf* gene with milk production traits and SCS.

The 5'-flanking region of bovine *Lf* gene is highly polymorphic. Twenty-nine polymorphisms have been identified within a 2.2 kb 5' flanking region of bovine *Lf* gene in the Irish cattle breeds (Holstein Friesians, Jersey, Jersey-Friesian crossbreeds, Norwegian Red, Montbeliard and Norwegian Red-Friesian crossbreed) that some of them were found within transcription factor binding sites, including GATA-1 and SPI transcription factor sites (Daly et al., 2006; O'Halloran et al., 2009). In this study, we identified 3 combined genotypes within 379 bp region in the 5'-flanking region of bovine *Lf* gene in Holstein cattle at Esfahan province of Iran. Three SNPs have been previously identified (Daly et al., 2006; O'Halloran et al., 2009) in this region (-602 T>G, -600 A>G and -586 T>C). In addition, it has been proved that some of the SNPs of the *Lf* 5'-flanking region occur only in a few dairy cattle breeds. For example, polymorphisms in positions -926 and -915 have been found only in Holstein Friesian (HF), New Zealand HF and Montbeliarde cattle (Daly et al. 2006).

Our statistical analysis revealed that combined genotypes of three SNPs in 5'-flanking region of *Lf* gene were associated with fat percentage and tended to associate with protein percentage and milk yield. There were no significant associations between studied haplotypes and SCS. The associations of combined genotypes with milk production traits could be explained by the mapping quantitative trait loci (QTL) for these traits on bovine chromosome 22. Several studies have investigated segregating QTL with significant effects on milk production traits in BTA22 (Ashwell et al., 2004; Mosig et al., 2001; Rodriguez-Zas et al., 2002) and SCC (Heyen et al., 1999; Ashwell et al., 2004).

Kaminski et al (2006) found that SNP at position -32 (G/C) relative to transcription start codon site of bovine *Lf* gene was significantly associated with milk protein content, but is not related to SCC, in Polish Holstein-Friesian cows. According to the author mentioned, a G allele reduces the *Lf* gene expression, and encourages a lower somatic cell count in milk. On the contrary, C allele causes a Lactoferrin concentration to increase, leads to a stronger immune response and in effect to a higher SCC. Kaminski et al. (2008) reported that polymorphism at position +216 related to the higher milk protein yields. When it occurs along

with polymorphism in the growth hormone receptor gene, it can increase the milk yield and elevate the milk protein and fat yield (Kaminski et al. 2008).

An association between the bovine *Lf* gene polymorphism occurring in intron 6 and SCC reported that homozygous AA animals have a lower SCC than heterozygote AB (Wojdak et al. 2006). Investigations of the same polymorphism revealed contrary results as the genotype BB animals showed the lowest SCC and heterozygote AB was the highest (Sender et al. 2006). O'Halloran et al. (2009) found that the C to T transition at -586 was associated with higher SCS and our results revealed that C haplotype show higher SCS. Huang et al (2009) found significant association between the combined genotypes of three SNPs (-3440T>G, -3879\_3880insG, and -4432T>C) in 5'-flanking region of *Lf* gene and SCS in Chinese Holstein cattle. In addition to error, the inconsistent results may be induced from differences in the number of analyzed animals, statistical approaches taken (Vukasinovic et al., 1999) and the genetic backgrounds of the populations (Ge et al., 2003).

## CONCLUSION

In the present study, through PCR-SSCP, we have identified three distinct haplotypes in the amplified fragment of 5'-flanking region at bovine *Lf* gene. Association analysis with milk production traits and SCS demonstrates different haplotypes significantly associated with fat percentage. Therefore, the result of this study showed that the bovine *Lf* gene is either a major gene that influences the milk fat percentage in Holstein cattle or is in close linkage with such a gene. Before applying these results for marker-assisted selection, additional studies are required to confirm the significant effect observed in this population.

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