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Association of genotypes at promoter region of lactoferrin gene with mastitis in crossbred cattle

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ABSTRACT

Mastitis inflammation, a cellular reaction to the irritant occurs. Lactoferrin is a component of the natural protection systems of human and animals. We investigated whether lactoferrin gene polymorphism is associated with mastitis. Blood samples were collected from 124 HF crossbred cattle under lactation from organized farms and field from Bengaluru district of Karnataka. The animals were examined for the presence of mastitis using California mastitis test, and separated into mastitis positive and mastitis negative groups. Oligonucleotide primers were used to amplify a 1049 bp long fragment corresponding to the promoter Lactoferrin locus. The amplified products were subjected to PCR-RFLP technique using *Hinf1* restriction enzyme. The *Hinf I* digestion produced 2 genotypes. The frequency of genotype AA was higher in mastitis free group, while the frequency of genotype BB was higher in mastitis group. Nucleotide sequence was submitted to the NCBI and released on public data base (Accession number HQ676491.1). The study revealed the polymorphism at the Lactoferrin gene promoter locus, can be used as a marker of susceptibility/resistance to mastitis in dairy cattle.

Key words: Crossbred cattle, Lactoferrin gene, Mastitis, PCR-RFLP, Polymorphism, Promoter

Inflammatory conditions of the udder (mastitis) represent a major problem in dairy cattle management. Mastitis is the single most important cause of financial loss to the dairy industry worldwide, with production losses amounting to more than $US 2 billion annually (Kossaibati and Esslemont 1997), and is believed to be influenced by many genes and numerous environmental factors (Wells *et al*. 1998). Dairy farm profitability is directly affected by mastitis, mainly due to decreases in milk production, increased veterinary intervention and labour costs, loss of income due to the need to withhold milk during treatment of clinical cases, and premature culling of affected animals (Petrovski *et al.* 2006). Genetic markers, used to measure the genomic response to selection in livestock were also found associated with changes in somatic cell count (Ashwell *et al*. 2004). Genetic improvement of milk production traits has accompanied an increased susceptibility to mastitis.

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Lactoferrin, a key role player in the defense mechanisms of the mammary gland of lactating animals, is synthesized mainly by breast epithelial cells and neutrophils (Plaffi *et al*. 2003). Bovine Lactoferrin in the apo-form showed bacteriostatic activity against mastitic *E. coli* (Rainard 1986).

Hirvoen (1999) reported that concentration of lactoferrin in milk and serum, changes in mastitis affected cows. Lactoferrin has several biological functions and among them a special attention is being paid to its antibacterial (Gonzalez-Chavez *et al*. 2009), antiviral (Pan *et al*. 2006, Mistry *et al*. 2007, Gonzalez-Chavez *et al*. 2009), antitumor and immunomodulatory properties (Gonzalez-Chavez *et al*. 2009). The objective of this study was to determine the lactoferrin polymorphism and association with mastitis.

MATERIALS AND METHODS

Randomly selected crossbred HF (124) cows were tested for mastitis using California mastitis test, and assigned into 2 groups, mastitis positive and mastitis negative. Blood samples (10 ml) were collected from each cattle with using EDTA as anticoagulant. The isolation of DNA from the whole blood sample was performed adopting the high salt method (Miller *et al*. 1988). PCR amplification (Chang-Hong Zhao

et al. 2009) of the 1049 bp long promoter region of Lactoferrin gene was carried out by using the primers, 5′-CACATTACAAGCAGGATCTTTTGCTG-3′ Forward and 5′-CTGGCCAATGAGCCCTATATGTGT-3′ Reveres. Amplification was carried out with initial denaturation of template DNA at 94°C for 4 min followed by 35 cycles of denaturation of 94°C for 30 sec, annealing temperature of 61°C for 45 sec and extension at 72°C for 45 sec. A final extension of 72 °C for 10 min was kept. Each ml PCR reaction consisted of 100 ng of genomic DNA, 20 p.mol of each primer, 750 mM dNTPs, 2 units of *Taq* polymerase and 5 ml of 10X assay buffer with 15 mM $MgCl_2$ in a reaction mixture of 50 ml.

The amplified products were digested with restriction enzymes. Each enzyme (1 ml) was used for the digestion of 5 ml of PCR product and incubated at 37 °C for 16 h digested products were analyzed electrophoretically in 2% agarose gel in TBE buffer. The samples which showed different RFLP patterns were selected for sequencing. Accession number HQ676491of present study released on public for analyzing the DNA sequence of promoter lactoferrin gene with other sequences in genomic. The observed genotype frequencies based on RFLP patterns were tested for Hardy-Weinberg equilibrium by Chi-square test.

RESULTS AND DISCUSSION

The PCR reactions were set up for the amplification of the promoter region from - 466 to–1515 region of bovine lactoferrin gene using the oligonucleotide primers. The amplification product was 1049 bp in length and found to be consisting in all the HF crossbred in 2 groups studied. The RFLP pattern of the bovine promoter Lactoferrin locus was generated by using *Hinf I* enzyme. The fragments promoter Lactoferrin were found 2 homozygotes with genotype BB and genotype AA revealing PCR-RFLP across all HF crossbred screened (Table 1).

Table 1. Genotypes and fragments of promoter lactoferrin gene in two groups digested with *Hinf I* RE in HF crossbred

Groups	Fragment size (bp)		Number of animals		
	AA	BB	AA	BB	(62+62=124)
Mastitis free	560, 489	585, 464	46	16	62
Mastitis	560, 489	585, 464	24	38	62

The gene frequency of mastitis free group for A and B alleles were 0.7419 and 0.2581, respectively, and those for mastitis group were 0.3871 and 0.6129 respectively. The observed frequency of genotype AA was higher in mastitis free group, while the frequency of genotype BB was higher in mastitis group (Table 2).

In the present study 1,049 kb promoter Lactoferrin gene was sequenced in HF crossbred, in each genotype. The BLASTn analysis of the nucleotide sequence compared the sequence AA genotype of promoter Lactoferrin gene sequences HF crossbred with accession # AY319306.2 (Zheng *et al*. 2005). Comparison revealed the occurrence of 12 nucleotide variations. Out of these 12 variations, 1 was a deletion (-/A), 5 were transitions (A/G, G/A, T/C, A/G and C/T) and the remaining were transvertions (T/A, C/A, C/G, A/C, C/G and C/A).

The AA genotype sequence analysis indicated that there is high homology between the present result and the published bovine Lactoferrin sequences. The nucleotide sequence AA genotype of present study revealed 91 to 98% identity with the subject *Bos taurus* with different accession numbers. The sequence AA genotype HF crossbred has revealed 90 to 91% identity with *Ovis aries,* 91 to 92% identity with *Muntiacus muntjak,* and 90% identity with *Capra hircus*. BLASTn programe analysis showed that the length adjustment was 33 bp and effective length of query was 1016 bp.

The present investigation was aimed at studying the genetic variation at promoter region of Lactoferrin gene in HF crossbred of cattle and to assess the possibility of developing a DNA marker system to reduce the incidence of mastitis. Lactoferrin is a multi-functional gene that exhibits many biological activities as reported. Due to the Lactoferrin relation to the innate immunity, this protein gene is supposed to be a promising candidate gene for the mastitis-resistance trait (Seyfert *et al*. 1996). Owing to these properties, Lactoferrin is considered as one of the most important factor that prevents and control mastitis in dairy cattle (Teng 2002).

In the present study, 1049 bp promoter lactoferrin gene amplified product was digested using the restriction enzyme

Table 2. Comparison of observed with expected frequencies of genotypes of promoter lactoferrin gene in HF crossbreds population

Genotype	Mastitis free group		Mastitis group		Allele frequency	
	Observed frequency	Expected frequency	Observed frequency	Expected frequency	Mastitis free	Mastitis
					A and B	A and B
AA	46	34.1	24	9.3	(A = 0.7419)	(A =0.3871)
AB	00	23.7	00	29.4	(B = 0.2581)	(B= 0.6129)
BB	16	4.2	38	23.3		
Total	62	62	62	62	62	62

Chi-square, df, 15.88, 1; P value, < 0.0001.

HinfI. BB genotype resolved 2 fragments of sizes 585 bp and 464 bp, whereas AA genotype resolved 2 fragments of sizes 560 bp and 489 bp. The presence of bands of size 464bp in BB genotype and that of 489pb in AA genotype corresponds with the report of Chang-Hong Zhao *et al.* (2008, 2009). However, these workers had also reported the bands of size 489 bp in BB and of 635 bp in AA genotypes. The present results are in complete contrast to this report. Since the total bps add up to the confirmed total of 1049bp, which has been confirmed by sequencing analysis (NCBI and accession number HQ676491.1 and DNA star software), the present work appears to be more authentic.

In contrast to the present research, Wojdak-Maksymieek *et al.* (2006) reported 2 alleles of Lactoferrin expected, A and B, were found in the studied population of dairy cows, their frequencies were 67.74% and 32.5 6%, respectively. The alleles controlled the occurrence of 3 genotypes – AA, BB and AB, with their frequencies of 37.90%, 2.42% and 59.68%, respectively. Restriction analysis of the amplified fragment was done with RFLP using *EcoRI* enzyme, the *Eco*RI digestion produced a mixture containing fragments of 301 bp.

Promoter is the key region for gene transcription. Its structure becomes a decisive factor for the starting frequency. Polymorphisms in these regions can alter gene expression. A fragment of size 1049bp of the total region of the promoter Lactoferrin gene was amplified by oligonucleotide primers, and confirmed with PCR-RFLP using restriction enzyme, *Hinf I*. The results revealed the existence of two homozygote genotypes AA and BB at promoter Lactoferrin gene locus. RFLPs were indicative of association between mastitis (susceptibility) or free mastitis (resistance) and promoter Lactoferrin gene. The presence of B allele with a higher frequency in the present study in mastitis group may probably indicate its association to mastitis, which needs to be further investigated.

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