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Original article

Lactoferrin gene promoter variants and their association with clinical and subclinical mastitis in indigenous and crossbred cattle

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Abstract

Lactoferrin (Lf) gene promoter was screened for the presence of single nucleotide polymphism in indigenous and crossbred cattle from North India and to evaluate its association with Mastitis. Study revealed the presence of genetic variation in regulatory region of bovine Lactoferrin gene using PCR-RFLP technique. Three genotypes namely GG, GH and HH were identified. A single nucleotide change, from guanine to adenine at 25th position was found to be significantly associated ($p<0.05$) with clinical mastitis in indigenous Sahiwal and crossbred Karan Fries cattle maintained at organised herd of National Dairy Research Institute, Karnal. A non-significant association was observed between subclinical mastitis, somatic cell score (SCS), and GG genotype in Karan Fries cattle, however, a lower SCS was observed in animals having GG genotype. Overall a lower incidence of clinical mastitis was recorded in those animals having GG genotype of Lf in Sahiwal and Karan Fries (KF) cattle. The SNP identified in the promoter region may effect expression lactoferrin protein, which may lead to different levels of antibacterial and anti-inflammatory activity of Lf gene. Results from this study indicated the probable role played by Lactoferrin promoter to serve as candidate gene for mastitis susceptibility among indigenous and crossbred milch cattle.

Key words: mastitis, lactoferrin promoter, cattle, single nucleotide polymorphism (SNP)

Introduction

Mastitis is an inflammatory condition, where the mammary gland and udder are affected in dairy animals. It is an endemic bacterial disease more prone to high yielding cows, causing high economic losses to the dairy sector. These economic losses include reduced milk production, discarded milk, increased cost of animal health care and labour at dairy farms. Lactoferrin (Lf) protein is considered to be a part of the innate immune system and variable concentrations are reported among healthy, subclinical and clinical

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mastitis, indicating that there is some association between Lf and mastitis, thus leading to change in expression of Lf gene in dairy cattle. Lactoferrin also takes part in specific immune reactions, but in an indirect way (Legrand et al. 2005). Lactoferrin protein affects the growth and a proliferation of a variety of infectious agents including Gram-positive and Gram-negative bacteria, viruses, protozoa, and fungi (Kirkpatrick et al. 1971).

Lactoferrin gene is located on long arm of chromosome 22 in bovines (Schwerin et al. 1994, Martin-Burriel et al. 1997), spanning about 34.5 Kb of genomic DNA. Lactoferrin protein is found in all external secretions including milk, tears, saliva and secondary granules of polymorphonuclear neutrophils (PMN) cells and helps in reducing bacterial load in target organ i.e. udder, eye etc. Researchers earlier showed that genetic variations present in bovine Lf gene can be associated with mastitis susceptibility (Bruckmaier 2005, Sharma et al. 2013, Chopra et al. 2014). Somatic cell refers to PMN cells, macrophages and lymphocytes along with small percentage of epithelial cells (Concha 1986). Somatic cell count in cattle milk had been positively correlated with subclinical mastitis and to lactoferrin content (Huang et al. 2010, Zabolewicz 2014)

Lactoferrin gene is supposed to be a candidate gene for the mastitis-resistance trait (Seyfert et al. 1996). Expression of bovine Lf mRNA is relatively low in the lactating gland but increases markedly during mammary gland infection (Molenaar et al. 1996, Bruckmaier 2005). This implies that there is a possible relationship between the bovine Lf gene and mastitis in dairy cattle. Polymorphisms in the promoter region of Lf gene may lead to different allelic variants, which can lead to differential expression of Lf protein and in turn leading to different levels of antibacterial and anti-inflammatory activity of Lf protein. Selection of animals with higher innate levels of antimicrobial proteins in their milk could be a solution for improving the health of dairy cattle suffering from mastitis. Therefore, the present study was aimed to explore genetic polymorphism of Lf gene promoter and to find out the association between clinical and subclinical mastitis with the allelic variants of Lf gene in Sahiwal and Karan Fries cattle.

Materials and Methods

Experimental animals: A total of 350 cows Sahiwal (n=200) and Karan Fries cattle (n=150) were considered in the study maintained at cattle yard of the National Dairy Research Institute, Karnal. All the cattle were screened for the presence of variations in Lf gene promoter. Study protocol was approved by institute local ethics committee.

DNA isolation and polymorphism detection: Genomic DNA was isolated from aseptically collected venous blood using the standard phenol/chloroform method with minor modifications (Sambrook and Russel 2001). NCBI sequence accession number AY 319306 was used to design the primer. Forward and reverse primers (PF 5'- GAAG-TCCTCCCCCACCCCTTGTCG-3' and PR 5'-AG-GACACTCCCTTGAAGCACAACA -3') with T_M of 69.6°C and 62.7°C, respectively, were designed using Primer-3 software. RFLP was done using *TaqI* restriction enzyme. Primers, restriction enzymes selected from MBI Fermentas, India and New England Biolabs, respectively, for a PCR amplified fragment size of 115 bp from promoter region of Lf gene. Optimization of PCR was done to get the best possible amplification of the product, at an annealing temperature of 580C for one minute. The detection results of allelic variation at SNP sites were based on the electrophoretic pattern of the restriction enzyme treated PCR products. Selected PCR products of all types of genotypes were sequenced using the automated dye terminator cycle sequencing method with *Ampli Taq* DNA polymerase in ABI PRIZM 377 DNA sequencer (Perkin – Elmer) and sequence comparison was made using CLUSTAL W (free available online software).

Mastitis incidence, statistical and genotype analysis: Data on clinical mastitis collected for all the screened cows were recorded from the treatment registers from organized herd of National Dairy Research Institute for the period of twelve year (2000 to 2012), and were used for further analysis. Genotype and allele frequencies were calculated and compared by gene counting method as suggested by Falconer and Mackay (1996). Association of Lactoferrin variants with affected and non-affected cows was calculated using Chi Square Test (JMP 5.0, SAS).

Somatic cell count analysis: Milk samples from 98 Karan Fries cattle were collected from three different stages of lactation namely, early (0-60), mid (60-180) and late (≥180) days, respectively for somatic cell count (SCC). Three different categories were considered based on SCC. Those animals showing SCC less than 2×10^5 were considered normal/not affected with mastitis, those between 2×10^5 – 5×10^5 were considered suffering from subclinical mastitis, those with SCC between 5×10^5 – 8×10^5 and greater than 8×10^5 were taken as suffering from clinical mastitis and chronic mastitis, respectively. Somatic cell counts were performed microscopically under 40X and 100X magnifications. Average No. of cells per field was multiplied by microscopic factor (8.811) to obtain

SCC (x 10^5) per ml of milk. SCC was transformed to somatic cell score (SCS) by using logarithms (Ali and Shook 1980).

$$SCS = Log_2 (SCC/100) + 3$$

Association between SCS and Lf genotypes: The log transformed SCS was analysed by linear model including fixed effects of genotypes, parity and stage of lactation (Harvey 1987).

$$\text{MODEL1: } Y_{ikl} = \mu + G_i + S_k + P_l + e_{ikl}$$

where, Y_{ikl} = SCS of l^{th} parity, k^{th} stage of lactation and i^{th} genotype; μ = overall mean; G_i = effect of i^{th} genotype on SCS (i = 1-3); S_k = effect of k^{th} Stage of lactation on SCS (k = 1-3); P_l = effect of l^{th} Parity on SCS (l = 1-5); e_{ikl} = random error associated with each observation assumed to be NID (0, $\sigma_e 2$).

The effects for different parity and season on SCS could not be classified therefore, linear model was modified, as shown below, with genotype as fixed effect.

$$\text{MODEL 2: } Y_i = \mu + G_i + e_i$$

where, Y_i = SCS of i^{th} genotype, μ = overall mean; G_i = effect of i^{th} genotype on SCS (i = 1-3); e_i = random error associated with each observation assumed to be NID (0, σ^2_e)

Results

Restriction enzyme linked polymorphism assay was done to screen indigenous cattle and crossbred cattle for the presence of SNPs (single nucleotide polymorphism). *TaqI* restriction enzyme was identified having recognition site in 115 bp fragment in promoter region, putatively affecting the expression of Lf gene. Lactoferrin promoter was found to be polymorphic in Karan Fries and Sahiwal Cattle. Three polymorphic patterns (Figs. 1, 2) with band sizes of 115, 91 and 24 bp, showing GH (115 and 91 bp), GG (91 bp) and HH (115 bp) genotypes in both Sahiwal (indigenous) and Karan Fries (crossbred) cattle were found. The homozygotes, GG and HH were found with frequency of 23% and 24% in Sahiwal and 8.67% and 40.67% in Karan Fries (Table 1). The heterozygotes (GH) were found with the frequency of 53% and 50.66% in Sahiwal and Karan Fries, respectively. The allelic frequency of **G** and **H** were 0.495 and 0.505 in Sahiwal, and 0.34 and 0.66 in Karan Fries, respectively. Sequencing results obtained for 115 bp amplicon (selected samples) revealed the SNP change from **G** to **A** (Fig. 3).

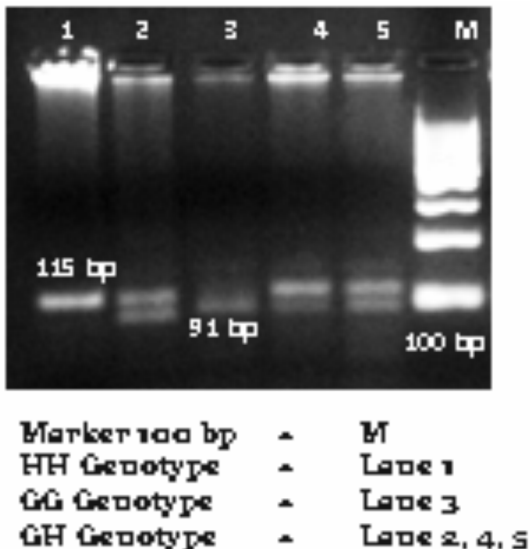

Fig. 1. PCR RFLP of lactoferrin gene promoter in Sahiwal cattle.

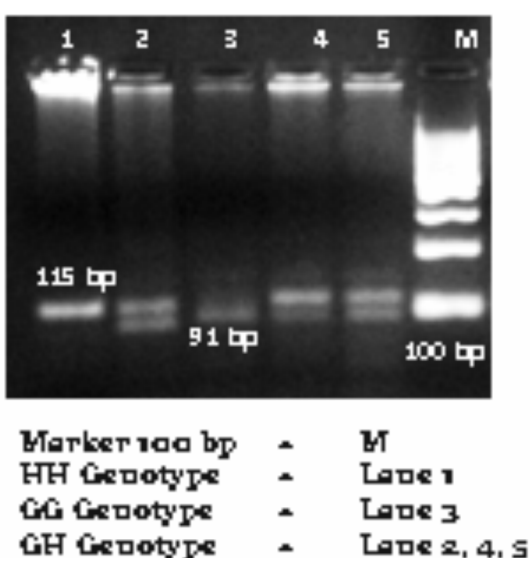

Fig. 2. PCR RFLP of lactoferrin gene promoter in Karan Fries cattle.

Incidence of clinical mastitis was found to be variable (Table 2); the highest for HH genotype followed by GH genotype and the lowest in GG genotype in Sahiwal cattle. The HH genotype in Karan Fries cattle showed lower incidence of clinical mastitis compared to Sahiwal cattle. These results when tested using Chi-square (Table 3) revealed significant association of Lf variants with the incidence of clinical mastitis with genotypes in both Karan Fries and Sahiwal cattle at 5% level of significance.

Effect of Lf genotype on subclinical mastitis was studied using somatic cell count in early, mid and late stage of lactation in Karan Fries animals. Observations recorded for three categories of the animals were classified based on SCC viz. not affected/normal,

Table 1. Genotype and allele frequencies of Lactoferrin promoter (115 bp amplicon) in Sahiwal and Karan Fries cattle.

Breed	Genotype	Number	Genotype Frequency (%)	Allele	Allele Frequency
Sahiwal	GG	46	23	G	0.49
	GH	106	53	H	0.51
	HH	48	24		
Karan Fries	GG	13	8.67	G	0.34
	GH	76	50.66	H	0.66
	HH	61	40.67		

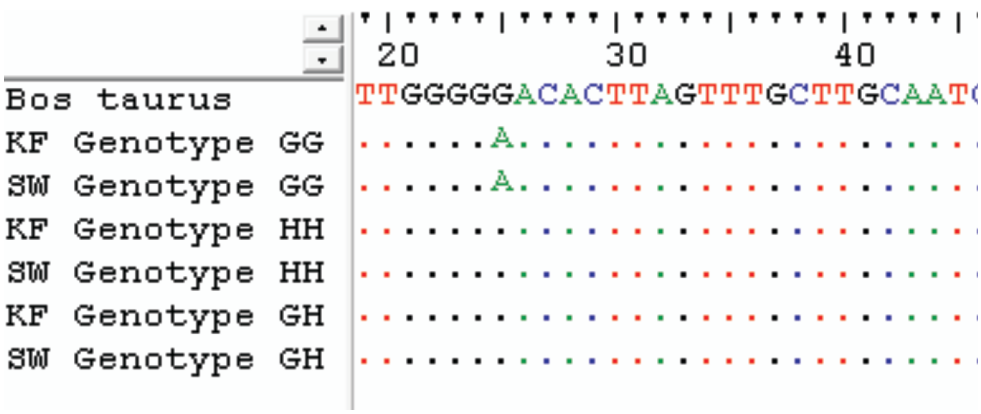

Fig. 3. Nucleotide sequence alignment using Clustal-W showing SNP change from Guanine to Adinine at 25th position in Lf promoter sequence in *Bos taurus* (Holstein Friesian), *Bos indicus* (Sahiwal) and *Bos indicus taurus* (Karan Fries).

Table 2. Mastitis Incidence for different genotype of Lactoferrin (Lf) promoter in Sahiwal and Karan Fries cattle.

Breed	Lf Variants	Mastitis not affected	Mastitis affected	Total	Mastitis Incidence (%)
Sahiwal	GG	29	17	46	36.96
	GH	53	53	106	50.5
	HH	17	31	48	64.58
Karan Fries	GG	11	02	13	15.38
	GH	48	28	76	36.84
	HH	50	11	61	18.03

Table 3. Association of different Lactoferrin variants with clinical mastitis in Sahiwal and Karan Fries cattle.

Cattle Breed	Genotypes	DF	χ^2 value	p-value
Sahiwal	GG GH & HH	2	7.194	0.0274*
Karan Fries	GG GH &HH	2	7.051	0.0294*

DF = Degree of Freedom; NS = Non Significant; * = P <0.05; ** = P <0.01

subclinical and clinical mastitis (Fig. 4). Under category classified as normal cattle the range of SCC varied from 1.32 to 1.98 hundred thousand per ml showing the minimum range of variation as compared to other two classes. However, the highest range of variation in PMN cells was found under chronic category, 9.04 to 14.90 hundred thousand cells per ml. Low range variations in SCC were present under sub clinical and clinical mastitis where the SCC range were 2.30 to 4.63 and 5.29 to 7.05 hundred thousand cells per ml, respectively. Model was run on log transformed SCS but not on SCC, as SCS trait showed the

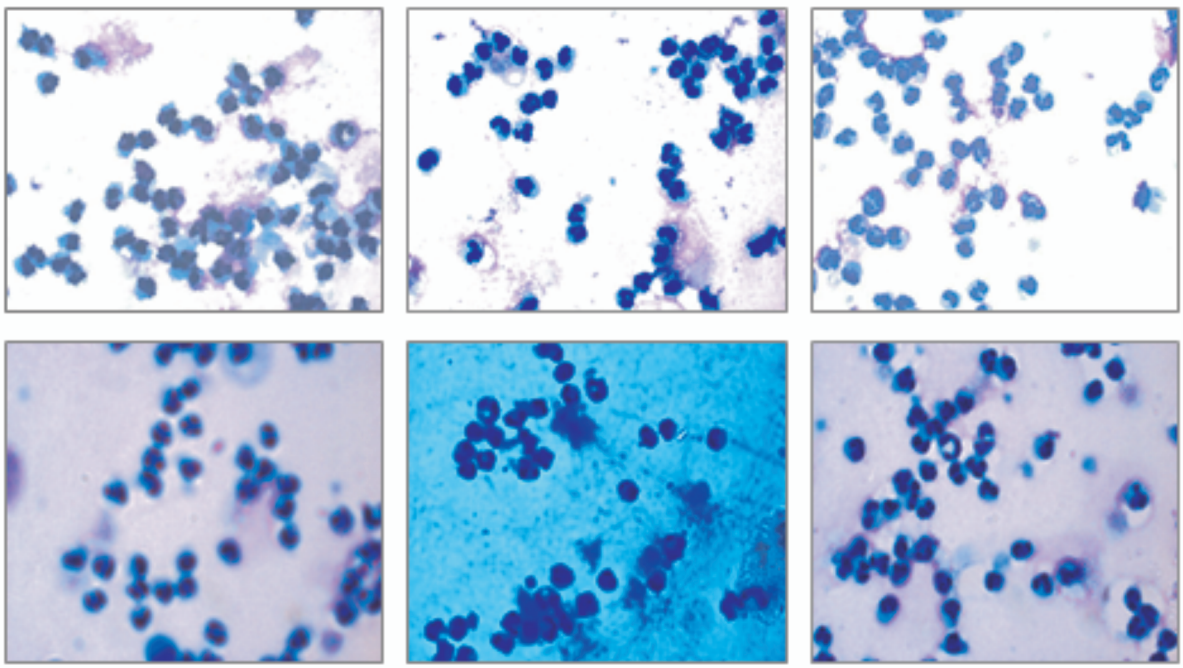

Fig. 4. Somatic cells in milk samples of Karan Fries cattle. x100

Table 4. Genotype and somatic cell score of Lactoferrin promoter in Karan Fries cattle.

Genotypes	Somatic cell score	Overall mean	F value	P value
GG	3.97 ± 3.80	7.32 ± 1.40	2.750.077^{NS}	
GH	10.64 ± 1.10			
HH	7.36 ± 1.31			

NS = Non Significant at $p<0.05$

normal distribution. The least squares mean SCS values indicated that GG genotype was showing lesser SCS in comparison to other genotypes in Karan Fries cattle. The overall results for least square mean for SCS values showed non-significant association at 5% level (Table 4).

Discussion

Variations and mutations in promoter region of lactoferrin gene play an important role in its transcription and regulation thus, an attempt to identify the SNP changes and allele diversity associated with mastitis susceptibility was studied in Sahiwal and Karan Fries cattle breeds. Two alleles (**H** and **G**) were identified with variable proportions in the samples studied. It was observed that the frequency of **G** allele was lower in comparison to **H** allele in Sahiwal and Karan Fries. Genotype frequencies for Lf promoter in Chinese Holstein cattle found by Huang et al. (2010) were partially similar to our results in Karan Fries cattle with almost similar genotype frequency of heterozygote GH and the results were different for homozygotes GG and HH. We found GG genotype less frequent in crossbred cattle whereas, Huang et al. (2010) reported HH genotype. In Sahiwal cattle, the GH genotype showed similarity with their results but on the contrary both GG and HH were having almost similar genotype frequencies. Other researchers have also reported polymorphism with different genotype frequencies in Lf promoter (Wojdak-Maksymieek et al. 2006, Chang-Hong Zhao et al. 2009) but not in the region we studied, except Huang et al. (2010). Therefore, conclusion could not be drawn, however, it was clear from the results that genetic makeup of crossbred cattle available abundantly in North India, is almost similar to that found in Holstein cattle. Probably because Karan Fries is a cross of Holstein Friesian male and indigenous Tharparkar breed. Our hypothesis is strengthen from the fact that frequency of these genotypes differ in pure indigenous milk cattle (Sahiwal) as compared to Holstein cattle. Selected samples of different genotype from Sahiwal and

Karan Fries were sequenced to identify the nucleotide change and confirm the RFLP results obtained. On comparing nucleotide sequences, in Sahiwal, Karan Fries and Holstein Friesian the **G** to **A** transition was found at 25th position. Similar results were reported by Daly et al. (2006) who found as much as 15 Single nucleotide polymorphisms (SNPs) in promoter region (~1100 bp) of bovine Lactoferrin gene across five cattle breeds including Holstein Friesian. Our results were also in agreement with Seyfert et al. (1994) indicating that Lf gene in bovines has sufficient variation present in promoter region and the animals have not been selected for this variation/marker for dairy herd improvement.

Harvey (1987) model can be used to study the variable effects of genotypes on SCS. The least square mean SCS values, in our study, indicated that GG genotype was showing lesser SCS in comparison to other genotypes in Karan Fries cattle. The overall results for least square mean values showed non-significant association at 5% level. Huang et al. (2010) reported no significant differences between one single SNP and SCS. However, significant associations ($p<0.05$) were observed using haplotype analysis of these SNPs. Cows with homozygotic genotypes had higher SCS compared to heterozygotic genotypes. These variable but non-significant results may be either due to lack of correlation between genotype and SCC or sampling error in our study. However, similar results were also reported by Li et al. (2004) who characterized bovine lactoferrin gene including 5'flanking region using PCR-SSCP and then examined its association with subclinical mastitis, but the effects of different genotypes to somatic cell count (SCS), pre-SCS and primary SCS which are considered to be indicator traits for subclinical mastitis have not got the significant statistic. Zabolewicz et al. (2014) have suggested significant association between SCC and Lf promoter genotype. To evaluate this association further in detail, a higher sample size could have been taken to reduce the sampling error. But considering lack of systematic data recording and small sized dairy farms in India, the population size taken for the present study can be considered optimum and these observations are of economic interest.

Polymorphism at the Lf gene promoter locus can be used as a marker of susceptibility/resistance to mastitis in crossbred dairy cattle (Rahmani et al. 2012). Germplasm of dairy breeds does vary between and within the countries of their origin, hence, efforts should be made to screen the native animals of dairy importance for the presence or absence of marker genotypes. Mastitis is global disease and is negatively affecting the dairy economics and India is no exception. Therefore, under the present study we measured the effect of Lf genotype on clinical and subclinical mastitis in major milk cattle breeds, which indicated that GG genotype of Lf promoter can serve as indicator or marker against mastitis susceptibility. Before applying this sort of genetic information in breeding and management decisions, studies with across the cattle populations are required to properly characterize the robustness of the associations of Lf promoter with clinical and subclinical mastitis.

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