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Association of Bovine Lactoferrin Gene with Mastitis in Frieswal Cattle

Research Article

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ABSTRACT

Thirty Frieswal lactating cows were screened for clinical and subclinical mastitis and subsequently classified into healthy, subclinically affected and clinically affected groups each group comprising of 10 cows. Polymorphism of cow lactoferrin (LTF) gene promoter was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The results showed that LFT gene promoter was polymorphic among different groups which indicated that its polymorphism was correlated to mastitis infection. The allelic frequency of C and G allele was 0.42 and 0.58 and they were associated with clinical and subclinical mastitis. It was found that LTF gene polymorphism and mastitis are highly associated with each other.

KEY WORDS LTF gene, PCR-RFLP, subclinical mastitis.

INTRODUCTION

Mastitis is a multi-factorial and costly problem affecting all milk-producing ruminants. The breed improvement programs have proven useful for the selection of milk production traits but are expensive and timeconsuming. Therefore, marker-assisted selection that supports fast and low-cost genetic progress and improves the accuracy of selection. In this regard, it is useful to study the genetic variations of candidate genes and their associations with milk production and somatic cell count (SCC) which have a high genetic positive correlation with mastitis (with an estimated average coefficient of 0.7) (Motwani, 2011). Daughters of sires that transmit the lowest SCC had the lowest incidence of clinical mastitis and the fewest clinical episodes during first and second lactations. The incidence of clinical mastitis and the number of clinical episodes per lactation may be reduced by selection for lower somatic cell score, longer productive life, shallower udders, deeper udder cleft, or strongly attached fore udders (Nash *et al*. 2000). Project directorate on Cattle, Meerut, India has developed a crossbred cattle "Frieswal" (5/8 Holstein Friesian and 3/8 Sahiwal) in collaboration with Ministry of Defense. Frieswal cows are being maintained at various military farms of the country. Presently the total population of Frieswal females at 37 military farms located in various agro-climatic regions of the country is 16874 that include1054 elite females. In a mature lactation of 300 days. Frieswal cows are producing 3542 kg of milk with 3.9 to 4.1% milk fat. (Lakshmi *et al*. 2010). The LTF, a ferric ion (Fe^{3+}) binding glycoprotein, is found most notably in milk (Guo-Hua *et al*. 2007). The bovine LTF gene, a member of transferrin gene family, spans about 34.5 kb of genomic DNA, consists of 17 exons. Higher concentration of LTF in milk has been associated

with lower occurrence of bovine mastitis. Some experiments have shown affinity of LTF with biomembrane. The antibacterial activity of LTF makes it a candidate gene for increasing resistance against infections of mammary gland (Seyfert *et al*. 1994). Some researchers reported that LTF concentration in milk and serum would change during the infection of mastitis which indicates that there is some association between LTF and mastitis. Therefore, observation on polymorphism of LTF gene by PCR-RFLP, and association between LTF and mastitis could give some novel insight into theory and practice (Zhao *et al*. 2008). In the present paper, polymorphism of LTF gene promoter and association between LTF and mastitis was investigated, by PCR-RFLP.

MATERIALS AND METHODS

Animals and experimental group

The quarter milk samples of 150 Frieswal lactating cows upto3rdlactation of similar nutrition, management conditions were screened for clinical mastitis by clinical examination of the udder as well as by strip cup test, and for subclinical mastitis by California mastitis test (CMT), (Schalm and Noorlander, 1957) and SCC. (Roger Mellenberger, 2000).

For screening the cows, SCC was determined in quarter milk samples using Fossomatic TM Minor cell counter (Foss Electric, Hillerod, Denmark) as recommended by the International Dairy Federation (IDF, 1984) and as described by Gonzalo *et al.* (2003). The SCC value more than 500 cells/µL of milk was considered positive for sub-clinical mastitis. The California mastitis test (CMT) was performed on quarter milk samples as per the method described by Schalm and Noorlander (1957). After screening the 150 cows by employing CMT and SCC, only 10 cows were found clinically positive and therefore a total of 30 Frieswal cows 10 in each group were classified as healthy, sub clinically and clinically affected.

Sample collection and DNA extraction

Blood samples were collected in vacutainer tubes containing ethylene diamine tetra-acetic acid (EDTA) (1 mg/mL). Genomic DNA was extracted using standard protocol (John *et al*. 1991) and stored at -20 ˚C until used for assay. The concentration of DNA samples was estimated using UV-visible range spectrophotometer and diluted to 30 ng/µL before PCR amplification. All the DNA samples were in the range of 1.8 to 2 at 260/280 optical density (OD), indicating high purity. The DNA was also examined by loading samples on 0.8% agarose gel and visualizing the band under UV light with a Gel Doc 1000 system (BioRad) after ethidium bromide staining.

Polymerase chain reaction

The polymerase chain reaction was based on the procedure reported by Zhao *et al*. (2009). Forward (5'-CAC ATT ACA AGC AGG ATC TTT TGC TG-3') and Reverse (5'CTG GCC AAT GAG CCC TAT ATG TGT-3') primers were used to amplify a 1143 base pair segment of the bovine LTF locus.

The reaction mixture consisted of 90 ng of template DNA, 10 pmole of each primer (1 µL each), 12.5 µL of 2X PCR mastermixjand 7.5 µL of DNAse free water. This solution was initially denatured at 94 ˚C for 10 min. followed by 35 cycles of denaturation (94 ˚C for 1 min), annealing (63 ˚C for 1 min), and elongation (72 ˚C for 1 min) and a final extension at 72 ˚C for 10 min. The PCR products were electrophoresed on 1.5% agarose gels in order to check the quality and specificity of DNA fragment amplification.

Restriction enzyme digestion

For PCR-RFLP analysis, the 1143 bp PCR products were digested with *HinfI* (*Fermentas Life Sci*.). Restriction fragments were separated by electrophoresis in a 2% agarose gel and their sizes were estimated using the molecular markers.

The results were taken into account when the sum of all the restriction fragments for *HinfI* enzyme was in the range of 1143 bp ±100. Ten µL of PCR products was digested for 12h at 37 ˚C with 1 µL units of restriction enzyme. Digested products were separated by electrophoresis on a 3% agarose gel and visualized with ethidium bromide under UV light with a Gel Doc 1000 system (BioRad) after ethidium bromide staining.

LTF gene polymorphic variants with somatic cell count

The SCCt for LTF gene was analyzed using simple analysis of variance model as given below:

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where:
Y_{ij}: mean $\log_{10}$ SCC of the j^{th} cattle for i^{th} genotype.
μ: overall mean.
G_i: effect of i^{th} genotype (i=1, 2, 3).
e_{ij}: random error.

RESULTS AND DISCUSSION

The quality and quantity of extracted DNA from analyzed samples was tested by electrophoresis on agarose gel where the DNA showed a single band. The DNA quantity was estimated using the molecular markers and the quantity for each band DNA sample was amplified and the PCR product was shown on agarose gel. As expected, the size of PCR

product of the LTF gene was 1143 bp in length (Figure 2) (Zhao *et al.* 2008).

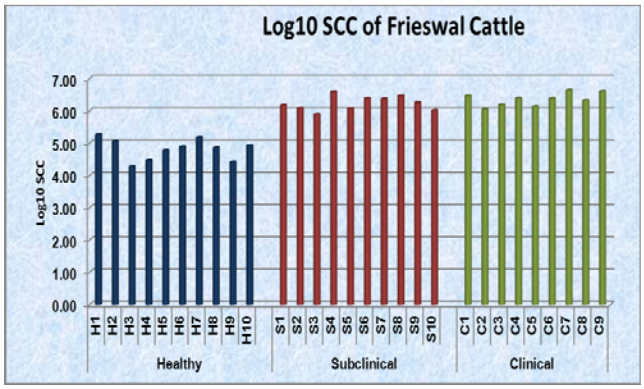

Figure 1 Log_{10} SCC of healthy and subclinical and clinical mastitis affected Frieswal cattle

Results of RFLP showed the existence of sequence encoding LFT in all sampled cows. Identification of different genotypes for LFT requires an enzymatic digestion. In this case, digestion was carried out by restriction enzyme *HinfI*, an endonuclease, which cuts at the restriction sites. Different fragments were separated by PCR products in LFT gene promoter using *HinfI*. Among 30 Frieswal dairy cows included in this study, 2 allele genes and 3 genotypes were revealed in each group. Allele gene (681 bp) was defined as A, whilst allele gene (462 bp) was defined as B. Other related genotypes were defined as G/G, G/C and C/C, respectively (Figure 3).

After digestion, allelic and genotypic frequencies were listed (Table 1).

Table 1 Frequencies of genotypes and alleles at LTF locus in Frieswal cattle

Genotype	Number of cows	Genotype frequency	Mean log_{10} somatic cell count
GG	5	0.17	6.23 (sub-clinical)
GC	15	0.50	6.23 (sub-clinical) 6.38 (clinical)
CC	10	0.33	4.81
Allele		Allele frequencies	
G		0.42	
C		0.58	
Chi-squre value		0.38^{ns}	

NS: non significant.

Among healthy dairy cows, CC genotype was detected with a frequency of 0.33, Frieswal dairy cows with subclinical mastitis showed GG (0.17) as well as GC (0.50) genotype and the cows with clinical mastitis showed GC genotype. The allelic frequency of G and C was 0.42 and 0.58 respectively.

The non significant Chi-square value in Frieswal cattle showed that the population was in Hardy Weinberg equilibrium.

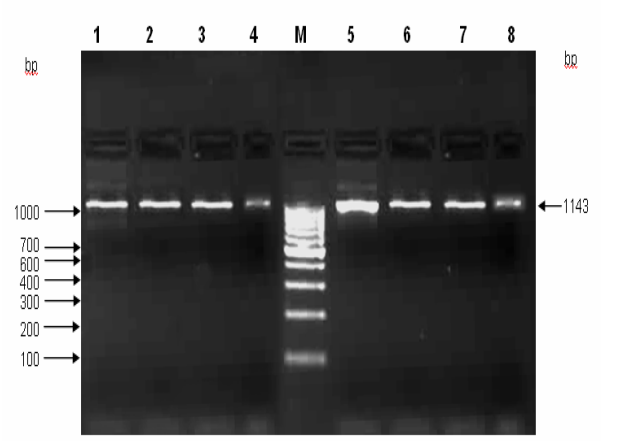

Figure 2 Amplified PCR product of LTF gene of Frieswal cattle electrophoresed on 2% agarose
M: 100 bp DNA ladder
Lanes 1-8: are amplified PCR product

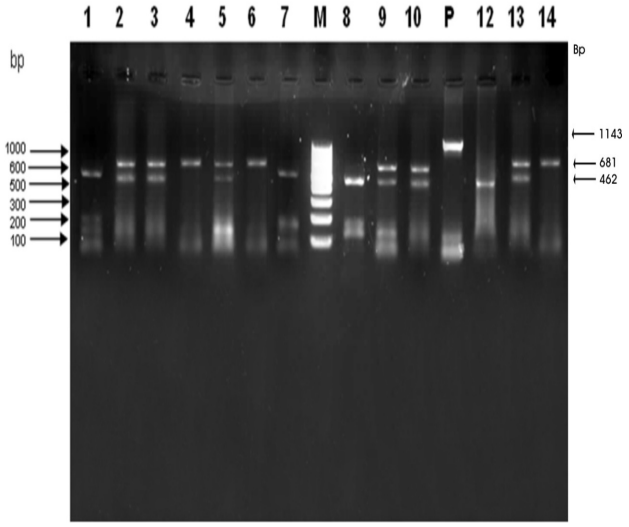

Figure 3 PCR-RFLP pattern of LTF gene digested with *HinfI* enzyme in Frieswal cattle
M: 100 bp DNA ladder
P: PCR product
Lane 4, 6, 14: GG genotype (681 bp)
Lane 2, 3, 5, 9, 10, 13: GC genotype (681, 462 bp)
Lane 1, 7, 8, 12: CC genotype (462 bp)

The association of SCC with LTF gene polymorphism was determined by analysis of variance on somatic cell count by using one way ANOVA. The relationship between genotypes and SCC was evaluated (Table 1).The LTF gene polymorphism showed significant association with SCC. The cows of genotypes GG and GC had higher SCC than those of the CC genotype.

LFT, a ferric ion ($Fe3^{+}$)-binding glycoprotein, is found most notably in milk and one member of transferrin family. Higher the concentration of LTF in milk, lower the occurrence of bovine mastitis. In the preliminary study of LTF, LFT was thought to act bacteriostatic agent due to its avid $Fe3^{+}$-ion binding capacity depriving growing microorganisms of their demands for ferric ions. With further research, it showed that the bactericidal and bacteriostatic mechanisms of LTF protein are very complicated. It can affect the integration of biomembrane in bacteria and results in the delivery of lipopolysaccharide from the membrane and increase the sensitivity of bacteria toantibodies and lysozyme (Guo-Hua Li *et al*. 2007). Wojdak *et al.* (2006) reported that homozygous individuals for A allele presented the lowest rates of somatic cells. Similar observations were reported by Zhao *et al.* (2008) indicating that cows with A/A genotypes were resistant to mastitis infection.

The obtained values of SCC were in lakhs which were further transformed to $\log_{10}$ SCC (Figure 1). The mean SCC value of healthy cows and those with subclinical and clinical mastitis were found to be 4.81 ± 0.08, 6.23 ± 0.07 and 6.38 ± 0.06, respectively. The analysis of variance on somatic cell count was calculated using the one way ANOVA. The relationship between genotypes and SCC was evaluated (Table 2).

Table 2 Analysis of variance table

Variable	Degree of freedom	Sum of Square	Mean square	F-value
Between genotype	2	15.107	7.554	110.51**
Within genotype	27	1.845	0.068	
Total	29	16.952	-	

** (P<0.01).

The LTF gene polymorphism showed significant association with the SCC. Cows of genotypes GG and GC had higher SCC than those of the CC genotype. The RFLP fragments and patterns of genotypes obtained in the present study are in agreement with RFLPfragments and patterns of genotypes as reported by Zhao *et al*. (2008) and Jemmali *et al*. (2011). Earlier, Xingping *et al*. (2007) studied association of Toll-like receptor 4 (TLR4) gene with mastitis and reported moderate polymorphism based on the value of polymorphism information content (*PIC*). The authors observed that cattle with allele A in T4CRBR1 exhibited lower SCC than in cattle with allele B which suggested possible role of allele A in imparting resistance against mastitis.

CONCLUSION

In the present essay, Frieswal cows were sampled to search polymorphism in the LFT gene. The SCC of milk samples and the PCR-RFLP of LTF gene was carried out by standard protocols. The results of PCR-RFLP showed three different band pattern in all samples implying three genotypes for the LFT locus. The Frieswal cows exhibiting RFLP pattern CC may have LTF genotype that are good genetic markers to indicate increased mastitis resistance while animals exhibiting GC and GG may have LTF genotypes as genetic markers for susceptibility to clinical and subclinical mastitis. The association between genotypes of LTF gene and somatic cell count was found to be highly significant (P<0.01). This can be used to design future studies to determine the association of LFT alleles with resistance to mastitis in Frieswal cows that may be used in genomic selection for breeding animals.

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