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Detection of allelic variants in lactoferrin gene promoter using created restriction site PCR-RFLP and its association with mastitis

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Dairy industry is suffering huge losses annually due to mastitis, which is one of the major production related diseases in cows. Mastitis is the single most important cause of financial loss to the dairy industry not only to India but worldwide and is clearly a global disease. According to an estimate loss due to mastitis approach $2 billion annually in the United States alone (Halloran 2009) and about ₹6,053.21 crore in India (Burman 2002). Lactoferrin protein is present in all external secretions including bovine milk and secondary granules of polymorpho nuclear cells and exerts several functions related to innate immunity and host defence. Level of lactoferrin protein in milk varies enormously between species and bovine milk is the most common source of commercially produced lactoferrin, concentrations ranging between 0.02 and 0.2 mg/ml in milk. Some reports indicated that lactoferrin (Lf) concentration in milk and serum would change during the infection of mastitis (Barkema 1998, Hirvoen 1999), suggesting that there was some association between Lf and mastitis. Therefore, observation on polymorphism of Lf gene by RFLP, and association between Lf and mastitis incidence could give some novel insight into theory and practice. In the present study, polymorphism of Lf gene promoter was investigated by created restriction site PCR-RFLP, and association between Lf genotypes and mastitis incidence was studied.

Experimental animals, DNA isolation and polymorphism detection: Genomic DNA was isolated from Sahiwal (200) and Karan Fries (150) cattle maintained at cattle yard of the Institute, using phenol/chloroform method (Sambrook and Russel 2001). NCBI sequence accession number AY 319306 was used to design the primer. Forward and reverse primers (PF 5'- AACCTACACATGCTGCAATGGAAG-3' and PR 5'-TGCTTATCGTTCACTGATTGCAGG-3') with T_M of 61°C, were designed using Primer-3 software with one of the primers containing a nucleotide mismatch, which enables the use of restriction enzyme for discriminating sequence variation. Restriction fragment length polymorphim genotyping was carried out using a tetra cutter restriction enzyme *Hae III* enzyme for a PCR amplified fragment size of 92 bp from promoter region of Lf gene. The optimization of PCR was done to get the best possible amplification of the product, at an annealing temperature of 59°C for 1 min. 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 automated dye terminator cycle sequencing method with Ampli Taq DNA polymerase in a DNA sequencer and sequence comparison was made using CLUSTAL W (free available online software).

Mastitis incidence, statistical and genotype analysis: Data on clinical mastitis was collected for all the screened cows, recorded in the treatment register maintained at organized herd of NDRI for a period of 12 years (2000 to 2012). Genotype and allele frequencies were calculated and compared by gene counting method (Falconer and Mackay 1996). Association of lactoferrin variants with affected and non-affected cows were calculated using Chi Square Test SAS EG 4.5.

Variations and mutations in Lf promoter may play an important role in the transcription and regulation Lf gene function. Created restriction site PCR-RFLP of bovine lactoferrin gene was conducted in Sahiwal and Karan Fries cattle. Polymorphism was observed in a 92 bp PCR amplified fragment. Three different genotypes were detected by 2.5% agarose gel with EtBr staining,viz. EE, EF and FF in Sahiwal cattle whereas the EE genotype was absent in our herd of Karan Fries cattle. In Sahiwal cattle EE EF and FF genotypes were having a frequency 2.5, 37 and 60.5% respectively. The frequency of E and F allele in Sahiwal cattle were 0.21 and 0.79 respectively. In Karan Fries cattle EF and FF genotypes were found with genotype frequency 14 and 86%, respectively. EE genotype was absent in Karan Fries cattle. The frequency of E and F allele in Karan Fries cattle were 0.07 and 0.93 respectively. Our results showed marked variations with Huang *et al.* (2010) and does not agree with respect to these alleles in Chinese Holstein Friesian cattle where they reported E and F alleles with

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Table 1. Association of different lactoferrin variants with clinical mastitis incidence in Sahiwal (n=200) and Karan Fries cattle (n=150)

Breed	Lactoferrin genotype	Non- mastitis cattle	Clinical mastitis cattle	Mastitis incidence (%)	Chi square value	P – value
Karan Fries	EF	14	7	33.33	0.428	0.5131^{NS}
	FF	95	34	26.35		
Sahiwal	EE	2	2	50	0.982	0.6121 ^{NS}
	EF	43	37	46.25		
	FF	54	62	53.45		

NS indicate nonsignificance at 5% level of significance.

0.546 and 0.454 frequencies, respectively. The marked variation may be attributed to the absence of EE genotype in Karan Fries cattle and intense selection carried out in NDRI herd. No parallel study is reported in indigenous Sahiwal cattle from India.

On sequence comparison of different genotypes of lactoferrin gene promoter in Sahiwal and Karan Fires cattle revealed synonymous variation at 67th position and T to G transversion was observed, E allele of Lf promoter in Sahiwal cattle showed guanine instead of thymine at this position. Daly *et al.* (2006) reported similar results as they found 15 single nucleotide polymorphisms (SNPs) in promoter region of bovine lactoferrin gene in animals of Holstein Friesian, New Zealand Holstein, Norwegian Red, Montebeliard and Normande cattle breeds. Our results were also in agreement with Seyfert *et al*. (1994). Halloran *et al.* (2009) reported 29 polymorphisms within a 2.2 kb regulatory region. Nineteen novel polymorphisms were identified and some of these were found within transcription factor binding sites, including GATA-1 and SPI transcription factor sites but none of these SNPs have been verified as a potential genetic marker for production traits in dairy cattle. However, Kaminiski *et al.* (2006) reported the polymorphism at +32 position in Lf gene promoter, which was significantly associated with protein yield and protein percentage in Polish HF cows.

Association of different lactoferrin allelic variants with incidence of clinical mastitis revealed the nonsignificant difference between 3 genotypes in Sahiwal cattle and nonsignificant association between the 2 genotypes with clinical mastitis incidence in Karan Fries at 5% level of significance using Chi-square test (Table 1). However, the incidence of clinical mastitis was variable; highest for FF genotype followed by EE genotype and lowest in EF genotype in Sahiwal cattle. The FF genotype in Karan Fries cattle showed lower incidence of clinical mastitis compared to Sahiwal cattle indicating that FF genotype is more susceptible to mastitis in indigenous Sahiwal cattle. It is noteworthy that heterozygote EF showed lesser incidence of mastitis in Sahiwal and higher incidence in HF crossbred Karan Fries cattle. Thus, it can be concluded that both E and F allele showed contrasting results with respect to mastitis incidence in Sahiwal and Karan Fries cattle. These variable but nonsignificant results may be due to sampling error, or due to the small sample size, high standard errors and imbalance data. Li *et al*. (2004) reported similar results while characterizing bovine Lf gene including 5'flanking region, and then examining their 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 did not get the significant level. Considering lack of systematic data recording and small sized dairy farms in India, the population size taken for the present study may be considered optimum and these observations are of economic interest. Although in this study we measured the effect of lactoferrin genotype on mastitis incidence in Sahiwal and crossbred Karan Fries cattle only, but before this sort of genetic information can be used efficiently in breeding and management decisions, studies with different populations are required to properly characterize the robustness of the associations of lactoferrin polymorphisms with clinical mastitis across cattle populations.

SUMMARY

Incidence of mastitis may be associated with single nucleotide polymorphism (SNP). Cows (350) belonging to Sahiwal and Karan Fries were screened. Polymorphism of bovine lactoferrin gene promoter was determined by using created restriction site polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Results indicated that lactorferrin gene promoter is polymorphic and showed varied levels of polymorphism among Sahiwal and Karan Fries cattle. Three genotypes were identified, viz. EE, EF and FF in Sahiwal cattle and EE genotype was absent in Karan Fries cattle. Association of SNP identified with incidence of mastitis using chi square test, revealed nonsignificant association with mastitis incidence.

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