Discussion
V. DISCUSSION

The present investigation is aimed at studying the genetic variation at promoter region of Lactoferrin gene in HF crossbred cattle and to assess the possibility of developing a DNA marker system for reducing the incidence of mastitis. The study also aimed at assessing the variations in nucleotide, gene expression and protein, if any, and to compare the same with earlier reports.

The agents that reduce the incidence of mastitis include Lactoferrin. Lactoferrin is involved particularly in the mechanism of alimentary immunity. This immunity results from the fact that possible infection factors have a limited availability of iron (as well as other growth agents, such as phosphorus and zinc), since its concentration in an organism's fluids is reduced.

The regulation of Lactoferrin differs from that of the other milk proteins. Lactoferrin is released from the specific granules of neutrophils, and thus the concentration of Lactoferrin in milk is also related to the number of neutrophils present in the milk (Hagiwara et al., 2003).

Lactoferrin is a multi-functional that exhibits many biological activities as reported in the review of literature. The Lactoferrin has important relation with the innate immunity, thus this protein gene is supposed to be a promising candidate gene for the mastitis-resistance trait (Pawlik et al., 2009; Seyfert et al., 1996). Due to its properties, Lactoferrin is one of the more important factors that prevent and control mastitis in dairy cattle (Seyfert et al., 1996; Hirvonen et al., 1999; Klungland at al., 2001; Teng, 2002).
5.1 Isolation of genomic DNA

The high salt DNA extraction procedure (Miller et al., 1988) was used with slight modifications for the isolation of high molecular weight DNA from blood samples. This procedure was also found to be suitable for purification of DNA from blood samples of sheep (Montgomery and Sise, 1990; Cushwa et al., 1996) and cattle (Gelhaus et al., 1995). Using the same procedure, in this laboratory, good results were obtained in sheep (Sunil kumar, 2010) and cattle (Vinoth kumar, 2009; Babu, 2010).

5.1.1 Yield and quality of DNA

About 300 µg of pure genomic DNA could be extracted from 10 ml of blood sample by high salt DNA extraction procedure. Montgomery and Sise (1990) reported higher yield of 640 µg DNA from sheep with high salt method.

Aravindakshan (1997) reported pure DNA in the range of 246 µg to 572 µg with an average of 360 µg per 10 ml of cattle blood and 300 µg to 707 µg with an average 452 µg per 10 ml of buffalo blood. By the use of same protocol, Nagaraja (1998) extracted about 400 µg of pure genomic DNA from 10-15 ml of cattle blood samples. The OD 260/280 ratio between 1.7 and 1.9 for most of the DNA sample indicated their purity. Thus, the high salt method is confirmed to be a very suitable procedure for DNA extraction in dairy cattle.
5.2 PCR-RFLP of promoter Lactoferrin gene

5.2.1 Amplification of promoter Lactoferrin gene by PCR

It is essential to optimize the different parameters for specific amplification of the target sequence in the DNA and for obtaining specific quantity of the desired product.

The different parameters such as annealing temperature, extension time and primers which play important roles in amplifying the desired product were optimized for amplification of the Lactoferrin from promoter region of the bovine Lactoferrin locus. The PCR protocol comprised of initial denaturation at 94 °C for 4 minutes. This was followed by denaturation at 94 °C for 30 s, annealing at 61 °C for 45 s and extension at 72 °C for 45 s, which was repeated for 35 cycles. A final extension was carried out at 72 °C for 10 minutes. Annealing temperature and time optimized in this study was 61 °C for 45 seconds for 35 cycles. This is in accordance with the work of Chang-Hong Zhao et al. (2009) who had also used 35 cycles of 61 °C for 45 seconds for optimum annealing, to obtain good amplification.

5.3 Promoter Lactoferrin gene polymorphism

5.3.1 Promoter Lactoferrin gene

The promoter Lactoferrin locus of the bovine has a sequence ranging from – 1 to – 4355 bp. Of this a 1049 bp fragment, the region from – 466 to – 1515 bp, was amplified by PCR using oligonucleotide primers. The size of amplification product was found to be the same for all the animals studied. The size of the amplified product being
more than 1000 bp, the technique of primer walking was employed and searching with software DNA star confirmed the product size as 1049 bp.

However, this is in contrast to the study by Chang-Hong Zhao et al. (2008, 2009) in the Xinjiang Shihezi dairy cattle. Using PCR-RFLP, these workers had amplified a Lactoferrin gene product of 1143 bp. No other published reports on the region of DNA investigated in the present study are available in the literature. Highly contrasting results between these two studies gives much scope for further studies of the same DNA region in different breeds of cattle. Further, the present study is more confirmative than that of earlier authors, because in the present study the size of the PCR product was confirmed by DNA sequence analysis by following primer walking technique. Howere, the earlier workers tested the PCR product size in agarose gel alone with DNA molecular weight marker, but did not confirm with sequence analysis.

5.3.2 PCR-RFLP

PCR-RFLP technique was followed to identify the genetic variants of promoter Lactoferrin gene. Two restriction enzymes *HinfI* and *EcoRI* were used to digest the PCR product for detecting polymorphism.

5.3.2.1 Restriction patterns with *HinfI*

The genotype which resolved two bands of sizes 585 bp and 464 bp was classified as BB, and the genotype which resolved two bands of sizes 560 bp and 489 bp was identified as AA. The samples analyzed did not exhibit any other combination of different band sizes.
Among the 124 HF crossbreds studied in the two groups, in the mastitis free group, 46 were of AA and 16 were of BB genotypes with respective genotype frequencies of 0.7419 and 0.2581. The gene frequencies were also 0.7419 and 0.2581 for A and B, respectively. In mastitis group, 24 were AA and 38 were of BB genotypes with genotypic frequencies of 0.3871 and 0.6129 for AA and BB, respectively. The gene frequencies in this group were 0.3871 and 0.6129 for A and B respectively.

The statistical analysis of genotype frequencies in the mastitis and mastitis free group revealed that neither of the two groups showed Hardy-Weinberg equilibrium.

Statistically significant (****P ≤ 0.05) deviations were found in the analyzed population between the observed distribution of Lactoferrin genotypes and their expected distribution estimated according to the Hardy-Weinberg law. Although differences were observed in the expected frequencies of genotypes in the two groups, in the present study, however, no significant differences were observed between the observed and expected values for allelic frequencies in both mastitis free and mastitis groups.

In a similar study, Chang-Hong Zhao et al. (2008, 2009) reported that in experimental/subclinical group BB genotypes were significant, and for control group AA genotypes were significant. The presence of B allele with a higher frequency in the present study may probably indicate its association to mastitis, which needs to be further investigated.

In the present study, 1049 bp promoter Lactoferrin gene amplified product was digested using the restriction enzyme *Hinfl*. BB genotype resolved two fragments of sizes
585 bp and 464 bp, whereas AA genotype resolved two fragments of sizes 560 bp and 489 bp. The presence of bands of size 464 bp in BB genotype and that of 489 bp 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 to the total of 1049 bp, which has been confirmed by sequencing analysis (NCBI and Accession numbers. HQ676491.1; JF420836.1 and DNA star software, the present work appears to be more authentic.

5.3.2.2 Restriction patterns with EcoRI

In the present study, restriction enzyme EcoRI did not cleave the PCR product in any two groups of animals investigated and an intact 1049 bp fragment was visible without any digestion.

This is in contrast to the report of Seyfert and Kuhn (1994) who had earlier reported two alleles, A and B, in the Lactoferrin locus, which encoded three possible genotypes: AA, AB, and BB. The frequencies of the alleles were 0.755 and 0.245 for A and B respectively.

Wojdak-Maksymiec et al. (2006) had also reported two alleles, A and B, in a study on Polish Black and White dairy cattle, with frequencies of 67.74 % and 32.56 %, respectively, which were in slight deviation from the report of Seyfert and Kuhn (1994). Three genotypes were observed, AA, BB and AB, with frequencies of 37.90 %, 2.42 % and 59.68 %, respectively. These workers had performed restriction analysis of the
amplified fragment with RFLP using *EcoRI* enzyme, the *EcoRI* digestion producing a mixture containing fragments of 301 bp.

The absence of PCR-RFLP polymorphism with *EcoRI* digestion can only be attributed to the type of genetic material utilized in the present study.

### 5.4 Lactation number

Lactoferrin may be active in modulation and regulation of macrophages, lymphocytes and neutrophil function (Smith and Oliver, 1981). Due to its properties, Lactoferrin is one of the more important factors that could prevent and control mastitis in dairy cows (Seyfert *et al*., 1996; Hirvonen *et al*., 1999; Klungland *et al*., 2001; Teng, 2002).

The present study reveals that the incidence of mastitis was higher from fourth lactation onwards, in mastitis group. Overall, the incidence of mastitis appears to increase with increasing number of lactations.

An effect of lactation number, lactation stage and breed on mastitis was also reported by Schutz *et al.* (1994) and Cameron and Anderson (1993).

### 5.5 DNA sequence analysis of promoter Lactoferrin gene

A 1049 bp fragment covering the region from –466 to –1515 bp was amplified by PCR using oligonucleotide primers. The amplified products of the promoter Lactoferrin locus in HF crossbred were gel eluted, cloned and sequenced at Chromous Biotech Pvt. Ltd. Bangalore.
The sequence of AA genotype was submitted to NCBI for query and was accorded the accession # HQ676491.1. The BLASTn analysis of the nucleotide sequence compared the sequence AA genotype of promoter Lactoferrin gene sequences HF crossbred with accession # AY319306.2, which had been submitted by Zheng et al. (2005). Comparision revealed the occurrence of twelve nucleotide variations. Out of these twelve variations, one was a deletion (-/A in position 55/-1450), five were transitions (A/G in position 108/-1407, G/A in position 182/-1332, T/C in position 189/-1325, A/G in position 833/-681 and C/T in position 875/-639) and the remaining were transversions (T/A in position 20/-1494, C/A in position 183/-1331, C/G in position 204/-1310, A/C in position 520/-994, C/G in position 885/-629 and C/A in position 960/-555). All the sequences shared high homology to the published Bos taurus Lactoferrin sequences.

The nucleotide sequence of HF crossbreds revealed 91 to 98 per cent identity with the subject Bos taurus with different accession numbers. The sequence HF crossbred has revealed 90 to 91 per cent identity with Ovis aries, 91 to 92 per cent identity with Muntiacus muntjak, and 90 per cent identity with Capra hircus. BLASTn programe analysis showed that the length adjustment was 33 bp and effective length of query was 1016 bp.

The BLASTn analysis was conducted for the gene expression omnibus (GEO) of the promoter from -1…to -4355 bp including the approximate region of study from –466 to –1515 bp of the promoter Lactoferrin locus of HF crossbreds. The search
analysis with BLASTn programe showed that length adjustment was 32 bp, and effective length of query was 1017 bp.

The HF crossbred sequence revealed a per cent identity varying from 86 to 92 with *Bos taurus* with different accession numbers, and 88 to 96 per cent identity with *Ovis aries*.

Comparison was made between protein sequences promoter Lactoferrin gene crossbred HF with those of *Bos taurus* species submitted earlier. The search analysis revealed that length adjustment was 139 bp, and effective length of query was 910 bp.

The protein sequence of HF crossbreds in the present study revealed per cent identity varying from 73 to 89 with different accession numbers for *Bos taurus*. There was a per cent identity of 80 with *Ovis aries*.

The sequence of BB genotype was submitted to NCBI for query and was accorded the accession # JF420836.1 The BLASTn analysis of the nucleotide sequence compared this sequence with accession # AY319306.2. Comparison revealed the occurrence of five nucleotide variations. Out of these five variations, two were transitions (G/A in position 175/-638 and T/C in position 943/-1407), and the remaining were transversions (G/T in position 92/-555, T/G in position 530/-993 and A/T in position 1033/-1496). The nucleotide sequence of BB genotype revealed 91 to 99 per cent identity with the subject *Bos taurus* with different accession numbers. There was 90 to 92 per cent identity with *Ovis aries*, 91 to 92 per cent identity with *Muntiacus muntjak*, and 91 per
cent identity with *Capra hircus*. BLASTn programme analysis showed that the length adjustment was 33 bp and effective length of query was 1019 bp.

The BLASTn analysis was conducted for the gene expression omnibus (GEO) of the promoter from -1…to - 4355 bp including the approximate region of study from – 465 to – 1515 bp of the promoter Lactoferrin locus of HF crossbreds. The search analysis with BLASTn programe showed that length adjustment was 32 bp, and effective length of query was 1020 bp. The HF crossbred sequence revealed a per cent identity varying from 86 to 93 with *Bos taurus* with different accession numbers, and 87 to 95 per cent identity with *Ovis aries*.

Comparison was made between protein sequences of promoter Lactoferrin gene with those of *Bos taurus* species submitted earlier. The search analysis revealed that length adjustment was 140 bp, and effective length of query was 912 bp. The protein sequence of HF crossbreds in the present study revealed per cent identity varying from 73 to 89 with different accession numbers for *Bos taurus*. There was a per cent identity of 81 with *Ovis aries*.

The PCR-RFLP and sequences data provided evidence that the HF crossbred is polymorphic for Lactoferrin locus. The allelic/gene frequency of Lactoferrin /HinfI polymorphism for A was significant in mastitis free group, whereas B gene frequency was higher in mastitis group.

This is the first such study on HF crossbred in India. Further study is needed to clarify the role of the genetic variants of Lactoferrin gene, and to analyze the mRNA expression level of Lactoferrin gene when udder is infected with mastitis. Indeed, an
eventual goal in the genomic study of mastitis is to identify these biologically relevant genotype-phenotype associations and to apply them to resistance breeding practice in dairy cattle.

**Conclusion**

- 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 enzymes, *Hinf I* and *EcoRI*.

- 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.