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# Identification of *CXCR1* gene variants and their association with somatic cell count in HF crossbred and Deoni cattle

**Bhajantri Shankarappa, R Nagaraja, Yathish HM and SB Prasanna**

**Abstract**
The present study was carried out to explore the variants of *CXCR1* gene and their association with Somatic Cell Count (SCC) in Deoni and HF Crossbred cows. The *CXCR1*/*Bsa*I analysis identified two genotypes AA and AG with respective frequencies of 0.17 and 0.83 in HF crossbred and 0.21 and 0.79 in Deoni cows. Allelic frequencies were 0.59 and 0.57 for a allele, and 0.41 and 0.43 for G allele in HF crossbred and Deoni cows, respectively. Sequence analysis confirmed the presence of 469bp nucleotides in *CXCR1* gene fragment studied and revealed lack of variations in the restriction site of *Bsa*I enzyme in 'A' allele of both HF Crossbred and Deoni cows. Multiple sequence comparison of 'A' allele of HF crossbred and Deoni cows using "ClustalW" method revealed two transitions *viz*. C>T at position 295 and A>G at position 299 in Deoni cows compared to HF crossbred cows. The chi-square analysis revealed significant difference between observed and expected genotypic frequencies in both HF crossbred ($p$<0.01) and Deoni ($p$<0.05) cows indicating deviation of both populations from Hardy–Weinberg equilibrium. Further, Chi-square analysis did not reveal any significant association between the genotypes and SCC in HF crossbred cows. However, a significant association ($p$<0.01) was observed between the *CXCR1* genotypes and SCC in Deoni cows, where in majority of animals with AG genotype were mastitis free.

**Keywords:** *CXCR1*, crossbred, Deoni, SCC, cattle

## Introduction
Bovine mastitis, an inflammation of the parenchyma of mammary gland is caused predominantly by infiltration of teat with number of bacteria especially *Streptococcus*, *Staphylococcus* and *Escherichia* (Awale *et al.*, 2012) [2]. It is a major source of economic loss and creating havoc to the dairy industry Worldwide (Russell *et al.*, 2012) [16] as it reduces milk yield, alters milk composition and elevates treatment costs (Nash *et al.*, 2003) [9] and still remains the dairy industry's costliest disease (Francoz *et al.*, 2012) [4]. In India, the economic losses due to mastitis have increased about 115-fold in the last five decades and presently the loss due to mastitis is to the tune of Rs. 7165.51 crores per annum (Anon, 2012) [1]. Mastitis tolerance is being a threshold trait; the selection strategies are a little different from the conventional approaches for quantitative traits. Lack of phenotypic data on clinical mastitis underscores breeding programmes and hence heavily relies on Somatic Cell Count (SCC) as an indirect indicator for subclinical mastitis (Rupp and Boichard, 1999) [15].
Electrical conductivity (EC) would give useful information only about udder health status and is not reliable when used alone in the diagnosis of mastitis (Galfi *et al.*, 2015) [5]. California Mastitis Test (CMT) was developed to achieve a faster, but less accurate result, a simple, inexpensive cow-side test, providing a qualitative estimate of SCC in the foremilk of individual cows or quarters. CMT is often used as an indirect measure of mastitis (Sharma *et al.*, 2011) [20] but SCC is regarded as the gold standard technique to measure mastitis when compared to other methods like EC and CMT (Hamann *et al.*, 2002) [7].
There are number of candidate genes associated with mastitis tolerance/susceptibility. One among them is CXC Chemokine Receptors 1 (*CXCR1*). The *CXCR1* gene has been mapped approximately 90.3 cM from the centromere of *Bos taurus* autosome 2 (BTA2) and consists of 7 exons and 6 introns spanning about 7.5 kb. These loci are approximately 1.3 cM from the Natural Resistance-Associated Macrophage Protein (NRAMP)-1, a polymorphic gene related to immune function in cattle, humans and mice, indicating that this region of BTA 2 may be associated with immune function and disease resistance (Grosse *et al.*, 1999) [6]. This gene encodes 360 amino acids (350 amino acids in human and 351 amino acids in mouse) and has 3 exons with coding region of about 1083 bp.

The CXC Chemokine Receptors 1 (*CXCR1*) plays a pivotal role in inflammatory response and eventual activation of the innate immunity**.** The *CXCR1* is expressed on the surface of neutrophils (Proudfoot, 2002) [12] and interacts primarily with *CXCL8* (*IL-8*), the most potent chemo-attractant for neutrophils. The activity of *CXCR1* is strongly associated with the inflammatory response to gram-negative bacteria infections and consequently, *CXCR1* is a key player activating the innate immune response (Oviedo-Boyso *et al.*, 2006; Rainard and Riollet, 2006) [10, 13]. A SNP located in the *CXCR1* gene at position +777, is reported to be associated with subclinical mastitis, SCS, milk yield and neutrophil function (Youngerman *et al.*, 2004; Rambeaud and Pighetti, 2005) [21, 14]. Therefore, the bovine *CXCR1* gene is a potential candidate gene to explore the molecular markers of mastitis resistance, as this is a critical component of neutrophil migration to the mammary gland during mastitis. So the current study was undertaken with the objective to detect genetic variations of *CXCR1* gene and to associate them with SCC in HF crossbred and Deoni cattle.

## Materials and Methods

All the experimental procedures and plan of study were duly approved by the Institute's animal Ethics Committee of Veterinary College Hebbal, Bangalore, Karnataka.

### Cattle resource population

A total of 244 unrelated cows comprising of 152 Holstein Friesian crossbred and 92 Deoni cows were utilized for the present study. The Holstein Friesian crossbreds were selected from different villages of Bengaluru Rural and Ramanagara Districts and also from Department of Livestock Farm Complex, Veterinary College, Bengaluru. The cows of Deoni breed were selected from Livestock Research and Information Centre (Deoni), Hallikhed, Bidar and from different villages of Bidar District of Karnataka state.

### Grouping of animals

Milk samples collected from randomly selected lactating Holstein Friesian crossbred and Deoni cows were subjected for California mastitis test (CMT) (Schalm and Noorlander, 1957) [17], Electrical Conductivity (EC) (Shagufta *et al.*, 2016) [19] and somatic cell count (SCC) (Schalm *et al.*, 1971) [18]. Amongst these tests, SCC was found to have higher sensitivity and specificity hence, the animals were classified based on SCC into mastitis positive (100 and 19) and mastitis negative (52 and 73) in HF crossbred and Deoni cows, respectively. Historical evidence was considered from interviews with animal owners and from institute records. The cows which had never been affected by clinical mastitis during their productive life (obtained from health records) and tested negative for SCC (less than 5 lakh cells /ml) were kept in the mastitis free group. Whereas, the cows affected with mastitis at least once on the basis of history of animals, positive for SCC (more than 5 lakh cells /ml) were kept in the mastitis affected group.

### Isolation of genomic DNA

Genomic DNA was isolated from venous blood by high salt method as described by Miller *et al.* (1988) [8], with minor modifications. The concentration and purity of genomic DNA, so isolated, were determined using UV-spectrophotometer at 260 and 280 nm (Bio photometer plus, Eppendorf, Germany). DNA samples having the OD 260/280 nm ratio between 1.7 and 1.9 were considered as good and were utilized for PCR amplification. The concentration of DNA samples was adjusted to 50ng/µl and then stored at -20°C for further use.

### PCR amplification of CXCR1 gene

The published primers, forward F 5′ TTATCATCCGCCATTTCGTT 3′ and reverse R 5′ TATGCCCTGGTCTTCTTGCT 3′ (Pawlik *et al.*, 2015) [11] were used for amplification of Exon 8 region (469 bp) of CXCR1 gene in the present study. Amplification was carried out in a 25µl reaction mixture that contain 9.5µl of nuclease free water (NFW), 10µM each of forward and reverse primer, 1µl of template DNA (50ng/µl) and 12.5µl of 2x Red PCR Master Mix. The PCR amplification was carried in thermocycler (Bio-Rad with S1000, USA). These reaction mixtures were subjected to initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 50 sec and extension at 72 °C for1 min and final extension at 72 °C for 7 min. Amplification of 469 bp region of *CXCR1* gene was confirmed by 1.5% agarose gel electrophoresis and documented under UV transilluminator (Gel documentation system-Bio-Rad Molecular imager Gel Doc XR+, USA).

### Restriction digestion of amplified DNA and sequencing

PCR amplified products were subjected for restriction enzyme digestion using *Bsa*I enzyme (New England Biolabs) in a total volume of 29µl containing 10µl PCR products, 2µl of 10X assay buffer, 1 µl (10U) of *Bsa*I enzyme and 16µl autoclaved NFW. These reaction mixtures were incubated overnight at 37 °C for digestion followed by heat inactivation of *Bsa*I restriction enzyme. These restriction enzyme digested products were resolved for 1h in 1x TBE buffer on 1.5% agarose gel stained with ethidium bromide (Bangalore Genie) and observed under UV transilluminator. Based on the electrophoretic pattern of the restriction enzyme-treated PCR products, genotypes were identified.

The representative PCR amplified gene products showing different RFLP patterns were custom sequenced bi-directionally by outsourcing (Chromous Biotech Private Limited, Bangalore) and the resultant sequences were analysed using Meg Align module of DNA Star software by "Clustal W" method.

### Statistical analysis

The genotypes were detected by scoring the bands under gel document system. The gene and genotype frequencies of different fragments were estimated by standard procedure (Falconer and Mackay, 1996). Presence of studied population in Hardy Weinberg Equilibrium (HWE) and association of *CXCR1* gene variants with SCC was analysed using Chi-square test.

## Results and Discussion

### PCR-RFLP and sequencing analysis

The PCR amplification of the exon 8 region of *CXCR1* gene produced the expected size, 469 bp in all the genomic DNA samples of HF crossbred and Deoni cows. These amplicons when subjected to RFLP analysis using *Bsa*I restriction enzyme, two band patterns were obtained in both HF crossbred and Deoni cows (Fig. 1). One pattern showed two fragments of sizes 259 and 210 bp length was designated as AA genotype and the other pattern showed three fragments of

sizes 469, 259 and 210 bp was designated as AG genotype. Contrary to the present findings, three genotypes (AA, AG and GG) were identified in Polish Holstein dairy cows (Pawlik *et al.*, 2015) [11] and in Chinese Holstein, Luxi Yellow and Bohai Black cows (Zhou *et al.*, 2013) [22].

The allelic and genotypic frequency, observed and expected heterozygosities, and Chi-square value for the *CXCR1* locus are shown in Table 1. In the present study, genotypic frequencies of 0.17 and 0.83 were observed for AA and AG, respectively, in HF crossbred cows, whereas, the respective frequencies were 0.21 and 0.79 in Deoni cows. The allelic frequencies observed were 0.59 and 0.41 in HF crossbred and 0.57 and 0.43 in Deoni cows for A and G, respectively. The higher frequency AG genotype observed in HF crossbred and Deoni cows is in agreement with reports of Pawlik *et al.* (2015) [11] of AG genotype (0.52) in Polish Holstein dairy cattle. Further, the observed and expected heterozygosities were 0.828 and 0.485, respectively in HF crossbreds, whereas, the respective values were 0.782 and 0.476 in Deoni cows.

The Chi square test showed that there was significant difference between observed and expected genotypic frequencies in HF crossbred ($p<0.01$) and Deoni ($p<0.05$) cows that indicated the deviation of studied populations from Hardy–Weinberg equilibrium.

Sequencing of representative samples has confirmed the 469 bp for *CXCR1* gene fragment and it has revealed lack of variations in the *Bsa*I restriction site in 'A' allele of both HF Crossbred and Deoni cattle. Multiple sequence comparison of 'A' allele of Deoni and HF crossbred cows using ClustalW method has revealed two SNPs *viz*. C>T and A>G transitions at 295 and 299 positions, respectively in Deoni cows compared to HF crossbred cows (Fig. 2 and Fig. 3).

**Table 1:** Allelic and genotypic frequencies, observed and expected heterozygosities and chi-square values for *CXCR1* locus in HF crossbred and Deoni cows

| Breeds | Genotypic frequency | | | Allelic frequency | | $H_o$ | $H_e$ | $\chi 2$ Value |
|---|---|---|---|---|---|---|---|---|
| | AA | AG | GG | A | G | | | |
| HF Crossbred | 0.17 | 0.83 | - | 0.59 | 0.41 | 0.828 | 0.485 | 70.163** |
| Deoni | 0.21 | 0.79 | - | 0.57 | 0.43 | 0.782 | 0.476 | 38.020* |

**-Significant at $P<0.01$; *-Significant at $P<0.05$

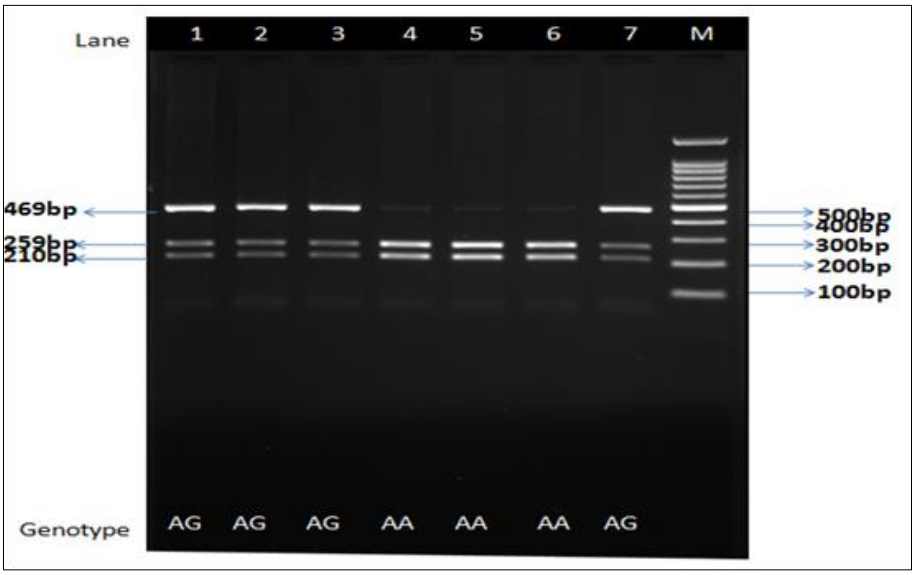
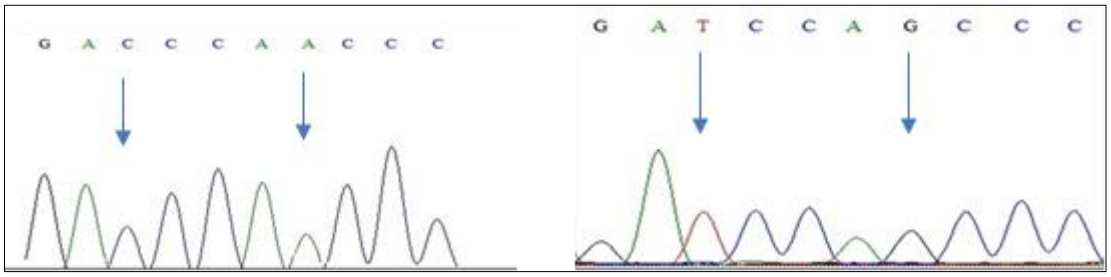



**Fig 1:** Agarose electrophoresis gel (1.5%) showing RFLP patterns of exon 8 region *CXCR1* gene in HF crossbred and Deoni cattle. Lanes 1-3: AG genotype and Lanes 4-5: AA genotype in HF crossbred. Lane 6: AA genotype and Lane 7: AG genotype in Deoni cows. Lane M: 100bp molecular marker

**Fig 2:** Multiple sequence alignment of 'A' allele of 469 bp fragment corresponding to exon 8 of *CXCR1* gene in HF Crossbred and Deoni cows showing two SNPs at 295 and 299 positions




'A' allele of HF crossbred cow 'A' allele of Deoni cow

**Fig 3:** Chromatogram showing C>T and A>G transitions at 295th and 299th positions in exon 8 of *CXCR1* gene

**Association of CXCR1 gene variants with SCC**

In HF crossbred cows, the Chi-square analysis did not reveal any significant association between the genotypes and somatic cell count (SCC). However, a significant association ($p$<0.01) was observed between the *CXCR1* genotypes and SCC in Deoni cows, where in majority of animals (90%) with AG genotype were mastitis free. Similarly, Pawlik *et al.* (2015) [11] have reported a significant ($p$<0.01) difference between *CXCR1* genotypes with somatic cell score (SCS) in Polish Holstein cattle. They revealed a higher test day SCS in animals carrying A allele (AA or AG genotype) than animals with G allele (GG genotype).

**Conclusion**

Genetic variants of *CXCR1* gene were observed in both HF crossbred and Deoni cows and the same was confirmed by the sequence analysis. However, these variants were not associated with Somatic Cell Count (SCC) in HF crossbred cows. Interestingly, the genotypes of *CXCR1* gene were significantly ($p$<0.01) associated with SCC in Deoni cows and it was inferred that Deoni cows with AG genotype had a lesser incidence of mastitis compared to those having AA genotypes. Therefore, AG genotype more specifically 'G' allele may be considered for selection of Deoni cows for lesser incidence of mastitis. However, this has to be validated in large population before its use in marker assisted selection.

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