



Genetic variation of *CXCR1* gene and its association with mastitis in Vrindavani crossbred cattle

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Mastitis is the most common and expensive disease of dairy cattle throughout the world. Major mastitis causing pathogens, which make serious disease in cow, include *Staphylococcus aureus*, *Streptococci* and coliforms. Inflammatory and immune responses are polygenic traits by nature and numerous studies have demonstrated statistical and functional associations between inflammatory diseases such as mastitis and polymorphisms in various immune-related genes (Youngerman *et al.* 2004, Leyva *et al.* 2007 and Ogorevc *et al.* 2009). The somatic cells in milk are primarily macrophages, neutrophils, and lymphocytes. These leukocytes play an important role in host resistance to intra-mammary infection (IMI). Neutrophils are the dominant cell type in alveolar milk, and during clinical mastitis they may represent more than 90% of the total mammary leukocyte population. The chemokine (C-X-C motif) receptor 1 (*CXCR1*) is expressed on the surface of neutrophils. Activation of *CXCR1* is believed to result in a wider array of antimicrobial processes than activation of *CXCR2* (Stillie *et al.* 2009). A single nucleotide polymorphism (SNP) located in the *CXCR1* gene at position +777, for example, is reported to be associated with subclinical mastitis, SCS, milk yield and neutrophil function (Youngerman *et al.* 2004). Therefore, the bovine *CXCR1* gene is considered as an excellent potential candidate marker for mastitis. In this study, we hypothesized that polymorphism in *CXCR1* gene contributes to resistance in mastitis. The objective of this experiment was to identify the SNP variants and to investigate the association between these gene variants and mastitis tolerance.

Unrelated Vrindavani crossbred cattle (130; Holstein Friesian/Brown Swiss/Jersey × Harijana) well maintained at Cattle and Buffalo Farm, IVRI, Izatnagar, were selected for this study. The cows which had never been affected by clinical mastitis during their productive life and tested negative for

California mastitis test (CMT) were kept in the mastitis unaffected group (74). Whereas, the cows affected with clinical mastitis at least once on the basis of history of animals and CMT during their productive life were kept in the mastitis group (56). Venous blood (15 ml) was collected from each animal in sterile polypropylene centrifuge tube containing 0.5 ml of 2.7% EDTA as anticoagulant. DNA was isolated by phenol-chloroform extraction method (Sambrook *et al.* 2001) and quality, purity, concentration was checked before applied for further analysis. A set of primers (5'-CTTCCGTGAGGCCTATCAAC3' as forward and 5'-AGGTCTCAGCAATCACATGG3' as reverse primer) was used on the basis of cattle genomic sequences to amplify a fragment (311 bp) corresponding to exon 2 of *CXCR1* gene. The reaction mixture and PCR programme were optimized to achieve the satisfactory level of amplification in a final volume of 25 µl containing 1µl of Genomic DNA (80–100 ng), 5 µl of 5X PCR buffer, 0.5 µl of dNTP mix (0.2 mM), 2 µl of MgCl₂ (1.5 mM), 1 µl each of forward primer and reverse (10 pmol/µl), 0.2 µl of Taq DNA polymerase (5U/µl). For *CXCR1* gene, samples were amplified for 35 cycles with initial denaturation at 94°C for 5 min, cyclic denaturation 94°C for 2 min, annealing at 60°C for 30 sec and extension at 72°C for 1 min followed by final extension at 72°C for 10 min. Amplified products were run on 1.5% agarose gel and a 50bp DNA ladder was run parallel to confirm the size of products. For single-strand conformation polymorphism (SSCP) analysis the PCR products were mixed with formamide dye, denatured at 95°C for 10 min and resolved on 12% polyacrylamide gel at 15mA for 8 h. Different conformation patterns were assigned as specific genotypes. The gene and genotype frequencies of different fragments were estimated by standard procedure. The PCR products showing different patterns were purified and cloned as per manufacturer's protocol. Samples were sent in duplicates as stab culture for sequencing. The sequences of the amplicons were submitted to GenBank (Accession numbers JQ410019 for allele A and JQ410020 for allele B). Resultant sequences were analyzed by DNASTAR programme with available

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sequences of different species at NCBI GenBank database. Analysis of data was done by statistical package SPSS-16 where $P < 0.05$ was taken as level of significance. The effect of season of calving, parity and *CXCR1* genotypes on mastitis of crossbred cows were taken into consideration for analysis. The following model was used for the analysis of the data;

$$Y_{ijklm} = \mu + P_i + S_j + X1_k + b(w_{ijkl(m)} - u) + e_{ijklm}$$

where, Y_{ijklm} , m^{th} observation for mastitis (1 for affected, 2 for unaffected) on the i^{th} individuals with respect to i^{th} parity, j^{th} season, k^{th} genotype of *CXCR1* μ , overall mean; P_i , fixed effect of the i^{th} parity ($k = 1-5$); S_j , fixed effect of j^{th} season of calving ($j = 1-4$); $X1_k$, fixed effect of k^{th} genotypes of *CXCR1* gene (k , 1 for AA, 2 for AB and 0 for BB); b , regression of milk yield (w) as covariate; e_{ijklm} , random residual error associated with m^{th} observation.

Neutrophils are a type of white blood cell (leukocyte), which forms an early line of defense against bacterial infections. They are highly responsive to interleukin-8 (IL-8), one of the critical chemokines in the immune system. IL-8 carries out its function by binding and activating its receptor, *CXCR1* which are located on the neutrophil cell surface. Combined analysis of Holstein families to detect quantitative trait loci (QTL) indicated that bovine chromosome 2 harbors a significant QTL for SCS. The *CXCR1* gene was mapped to *Bos taurus* autosome (BTA) 2 at 90.3 cM and is part of the gene family encoding the serpentine 7 transmembrane domain G-protein coupled receptors. The 1703bp long bovine *CXCR1* gene (GenBank Acc. No. NC_007300) contains no introns and the coding region is 1083 bp long and is translated into the *CXCR1* protein (GenBank Acc. No. NP_776785) of 360 amino acids. The annotation of *CXCR1* was corrected by Pighetti and Rambeaud (2006). A 311 bp fragment of *CXCR1* gene was amplified using the PCR primers (Fig. 1).

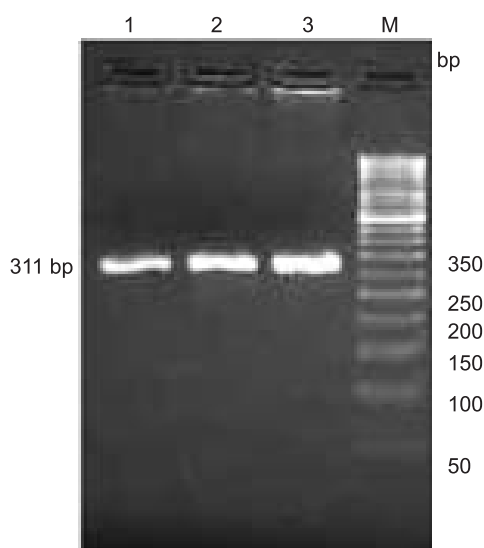


Fig. 1. PCR amplification of 311 bp fragment of *CXCR1* gene in 1.5% agarose gel. Lane M: 50 bp DNA ladder, Lane 1-3: 311 bp amplified PCR product.

PCR-SSCP produced 3 genotypes namely AB, AA and BB (Fig. 2) with frequencies as 0.48, 0.39 and 0.13, respectively, and the allelic frequencies as 0.63 for A, and 0.37 for B in mastitis affected cattle. Similarly, unaffected group also produced 3 genotypes having frequencies of 0.5 for AB, 0.39 for AA and 0.11 for BB and allelic frequencies as 0.64 for A and 0.36 for B.

The genotype AB and allele A were predominant in both the affected as well as unaffected groups of cattle. The results also suggested that there is not much variation in allelic frequencies of both the groups of cattle. Sequence analysis of this fragment also confirmed the results observed by SSCP. Both the alleles of the 311 bp fragment were compared with the sequences of *Bos indicus* (DQ389109, DQ389111), *Bos taurus* (HM013954), crossbred (DQ389113), *Bubalus bubalis* (AY864732), pig (XM_003133651) available at NCBI database. Holstein cows expressing the CC genotype at position +777 had increased incidence of subclinical mastitis (37%) compared to Holstein cows that expressed the CG (21%) or GG (22%) genotypes of *CXCR1* (Pighetti and Rambeaud 2006). In a study by Beecher *et al.* (2010), *CXCR1*-777 tended to associate with somatic cell score (SCS) in 246 lactating dairy cows. But, in the present study this SNP was not detected whereas the sequence alignment report revealed two SNPs at positions +563 (A'→T) and +812 (T'→A) in allele A compared to allele B. The sequence pair distance in Clustal V analysis showed the allele A of *CXCR1* gene has 99.4% similarity with allele B, and 96.1% with *Bos taurus*. Similarly, the results of the phylogenetic study suggested that the observed allele A and B had closer lineage

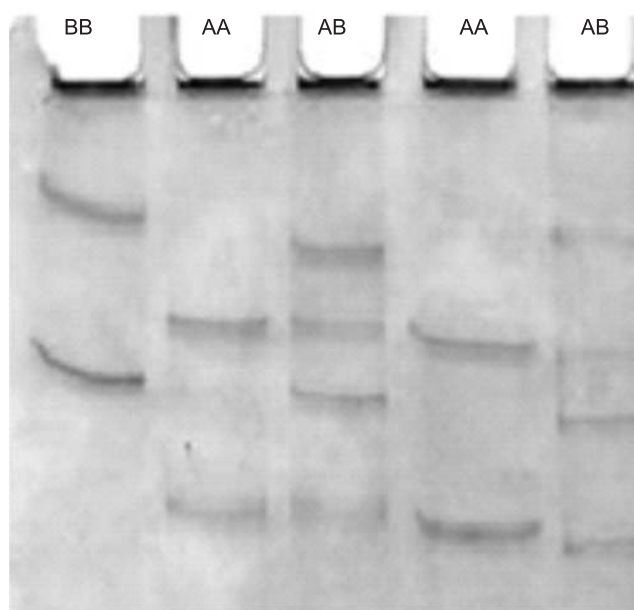


Fig. 2. 311 bp fragment (*CXCR1* gene) showing different PCR-SSCP pattern for different genotypes in 15% PAGE with a cross-linking ratio of 49:1 at 15mA for 8 h. Three patterns were obtained and designated as AA, AB and BB as shown in the figure.

with the reported allele of *Bos taurus* and *CXCR2* of *Bos indicus*. Our results showed that the effect of parity was significant ($P < 0.05$) on the cows belonging to clinical mastitis groups. Almaw *et al.* (2008) reported that the parity number 3–5 and late lactation stages increased occurrence of mastitis significantly ($P < 0.01$). The effect of season of calving was found non-significant on the mastitis occurrence in crossbred cows. The general linear model analysis in our study revealed that there is no significant effect ($P = 0.552$) of genotypes of *CXCR1* gene on the occurrence of clinical mastitis. Chen *et al.* (2011) analyzed the polymorphisms of *CXCR1* gene by PCR-SSCP and found association between different genotypes and somatic cell score (SCC) in Chinese Holstein cattle. On the contrary, in another study by Verbeke *et al.* (2012) *CXCR1* genotype was not associated with SCC in lactation. Reasons for the discrepancy between the results from previous reports and this study are not clear. A possible explanation might be sample sizes.

SUMMARY

CXCR1 plays a key role in mastitis resistance via IL8 signaling pathway. In the present investigation the genetic polymorphism of *CXCR1* gene was investigated but no significant association between the genotypes with the clinical mastitis was obtained. *CXCR1* was found to be polymorphic in both the affected as well as unaffected groups of cows which are confirmed after sequencing the allelic variants. Furthermore, these findings did not support the association of *CXCR1* alleles with resistance; hence associations of genotypes with clinical mastitis are to be verified in large population of crossbred cattle for further use in marker assisted selection. SNPs located within other regions of the bovine *CXCR1* gene may also affect gene function during mastitis infections which are needed to be addressed.

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