

PCR-SSCP and Sequencing of CXCR2 Receptor Gene in Vrindavani CattleDige Mahesh Shivanand^{1*}, S.P.S Ahlawat¹, Bharat Bhusan¹, A. K. Tiwari², Arvind Sonawane¹, Pushpendra Kumar², B Inamdar¹, Triveni Dutt³¹Division of Animal Genetics and Breeding, Indian Veterinary Research Institute, Izatnagar, Bareilly (Uttar Pradesh) - 243122 India.²Division of Veterinary Biotechnology, Indian Veterinary Research Institute, Izatnagar, Bareilly (Uttar Pradesh) - 243122 India .³Division of Extension Education, Indian Veterinary Research Institute, Izatnagar, Bareilly (Uttar Pradesh) - 243122 India

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Abstract

Genetic markers associated with inflammatory responses during mastitis could aid in the selection of diseased cattle. One potential marker is CXCR2, a chemokine receptor required for neutrophil migration to infection sites. The objective of this experiment was to identify genetic polymorphism of CXCR2 gene and associate it with subclinical and clinical mastitis. Ninety five Vrindavani crossbred cows (42-mastitis tolerant and 53-clinical mastitis) that completed at least two full lactations were taken for study. Blood of selected crossbred cows was collected, and genomic DNA was isolated by phenol chloroform method. The DNA of good quality having OD ratio (260/280 nm) between 1.7-1.9 were used for further analysis. PCR-SSCP technique was used to reveal the polymorphism in 269bp fragments of CXCR2 gene. The 269 bp fragment of CXCR2 gene was found to be monomorphic in all the DNA samples of crossbred cows.

Keywords: CXCR2 Receptor gene; PCR-SSCP; Vrindavani; Cattle; Sequencing

Introduction

Mastitis is the inflammation of the mammary gland caused by invading pathogens. Many candidate genes for mastitis tolerance are being studied in a variety of populations so as to find out the molecular markers. Majority of these candidate genes have one or multiple roles in the host immune system. Genes associated with immune responses of mammary gland are potential genetic markers because of their importance in mastitis. Besides, genes associated with neutrophil function are potential genetic markers for mastitis, as neutrophil migration from blood to the sites of infection is essential for resolution of most mastitis pathogens (Paape *et al.*, 2000). The ability of neutrophils to migrate into infected tissues is dependent upon recognition of inflammatory mediators by neutrophil cytokines, chemokines, and complementary receptors (Burvenich *et al.*, 1994).

One of the important chemokine associated with leukocyte migration is interleukin-8 (IL-8), which is an ELR+ CXC chemokines, that interacts with specific chemokine receptors viz. CXCR1 and CXCR2 present on the neutrophils surfaces (Lahaussa *et al.*, 2008). These chemokines receptors are required for maximum neutrophil function during infection (Murphy and Tiffany, 1991). Recognition of chemokines by CXCR1 and CXCR2 induces neutrophil activation, chemotaxis and eventually phagocytosis of pathogen (Peveri *et al.*, 1988, Podolin *et al.*, 2002). Being receiver and transmitter of the signal from IL-8 to downstream, the receptors of IL-8 are the important candidate genes for mastitis tolerance/susceptibility study in the herd. Single Nucleotide Polymorphisms (SNPs) have been identified in the bovine CXCR1 and is found to be associated with mastitis resistance. However, CXCR2 still needs to be explored for SNPs associated with mastitis resistance. In the present paper, PCR-SSCP is proposed to identify SNPs in CXCR2 receptor gene of cattle and associated it with mastitis resistance in cattle. PCR-SSCP method is easy, sensitive, effective and inexpensive

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to find out the SNPs.

Materials and methods

Experimental Animals

The present study was conducted on Vrindavani crossbred cows (crosses of Holstein Friesian / Brown Swiss/ Jersey with Haryana), maintained at cattle and buffalo farm, IVRI, Izatnagar. These cows were maintained under similar feeding and managemental practices. A total of 95 crossbred cows (42-mastitis tolerant and 53-clinical mastitis group) were selected for this study. The cows which had never been affected by clinical mastitis during their productive life and tested negative for CMT were kept in the mastitis tolerant group. Whereas, the cows affected with clinical mastitis at least once during their productive life were kept in the clinical mastitis group. The California mastitis test (CMT) was conducted during milking of cows as per method described by Schalm (1957).

Collection of Blood Samples and DNA Isolation

About 10 ml of venous blood was collected from each animal of both mastitis affected and non affected group under sterile conditions from jugular vein of each animal in sterile polypropylene vials containing 0.5 ml of 0.5M EDTA solution. The collected blood samples were mixed gently with anti-coagulant and then transported to the laboratory in a thermocol box containing ice and cool packs. The blood samples were kept at -20°C until the isolation of genomic DNA. Genomic DNA was isolated from the frozen blood samples following the phenol-chloroform extraction method given by Sambrook and Russell (2001). The purity of genomic DNA was assessed by spectrophotometry. All the samples yielded a sufficient amount of DNA. The isolated DNA samples were stored at -20°C for further analysis.

Polymerase Chain Reaction

A small 269 bp fragment of CXCR2 gene comprising part of intron and exon-2 region of CXCR2 receptor gene was amplified using a set of forward 5'-CAAGTTGTTGGGCTAGAATCTGGG-3' and reverse 5'-AGTTTCCCAGGAGGCTTAGCAAG-A-3' primers, designed with the help of online IDT (Integrated DNA Technology) Software. The 25 μl of PCR reaction mixture was prepared using 20

pmoles of each primer, 200 μM of each dNTPs, 1.5 mM MgCl_2 , 5 μl of 5X PCR assay buffer, 80-100 ng DNA template and 1 U Taq DNA Polymerase. The amplification was carried out using a pre-programmed thermal cycler (PTC-200, M.J. Research) with the following conditions: Initial denaturation of 4 min at 94°C , followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 30 sec and extension at 72°C for 30 sec and finally the final extension of 10 min at 72°C . The PCR products were checked by agarose gel electrophoresis using 1.5% agarose gel in 1X TBE buffer at 6 volts/cm for one hour. The amplified product was visualized under UV transilluminator and documented under gel Documentation System (Fig. 1).

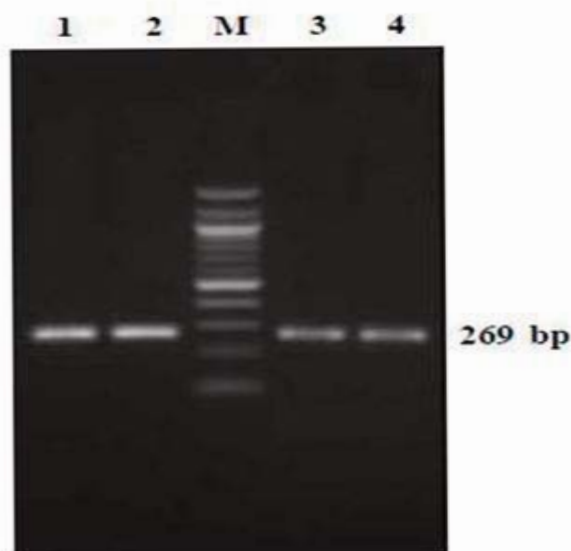


Fig. 1. PCR product of 269 bp fragment of CXCR2 gene. Lane M: 100bp DNA ladder. Lane 1-4: Amplification of 269bp fragment

Single Strand Conformation Polymorphism Analysis

Single Nucleotide Polymorphisms (SNPs) were screened in this fragment using SSCP technique (Orita et al., 1989). The PCR products were resolved on 15% polyacrylamide gel. 50 ml of PAGE solution was prepared by adding 15 ml of Acrylamide: Bis-acrylamide (50: 1), Autoclaved Triple Distilled water 29.6 ml, 5 ml of 10 X TBE, 330.001 of 10% Ammonium persulfate and 70.0 μl of TEMED. After thorough mixing, the freshly prepared PAGE gel mix was poured into the space between plates and spacer. Then the comb was inserted immediately with care so as to leave no air bubble inside the gel. Then the gel was allowed to

polymerize at room temperature for 1 h and was given a pre-run at 200 V for 60 minutes in a vertical gel electrophoresis system. About 5 ul of PCR product was taken in a 0.2 ml PCR tube and 15 ul denaturing formamide dye (Formamide, 95%; Xylene cyanol, 0.025%; Bromophenol blue, 0.025%; 0.5 M EDTA, 4%) was added and mixed properly. The mixture of PCR product and formamide dye were denatured at 95°C for 5 minutes and snap chilled on ice for 15 minutes. The product was loaded in gel carefully. The electrophoresis was performed at 4°C temperature at 130v for 18 hours. After running, the gels were silver stained.

Silver Staining

For visualization of bands, silver staining was carried out as per the method described by Bassam (1991) with appropriate modifications made to suit this fragment in our laboratory conditions. The gel was agitated in 400 ml of 10% glacial acetic acid slowly for 45 minutes or until the tracking dye was no longer visible. The gel was rinsed for 3 times in double distilled water for 5 minutes each. 400 ml of 0.1% silver nitrate solution was prepared and to it about 450 .00l of 37% formaldehyde solution was added just 15 minutes before adding the solution to the tray and agitated slowly for 30 minutes, fol-

lowed by brief rinsing in distilled water for 30 second. Subsequently, the gel was developed in 400 ml of 3% sodium carbonate having 600 ul of formaldehyde solution. The reaction was stopped by adding 400 ml of 10% glacial acetic acid. The gel was visualized and documented under gel documentation system.

DNA Sequencing

The purified PCR product was sequenced using the automated Sanger's dideoxy chain termination sequencing method in automated ABI Prism DNA sequencer. The sequence obtained from different animals was subjected to BLAST analysis to ascertain that sequences were of CXCR2 gene.

Results

A small 269 bp fragment of CXCR2 gene was amplified (Fig. 2), followed by SSCP analysis. The SSCP analysis of 269 bp fragment of CXCR2 gene revealed same patterns in all samples of cattle. Thus only one genotype was found for this fragment of CXCR2 gene. A 269 bp fragment of CXCR2 gene was sequenced by Sanger's dideoxy chain termination sequencing method in automated ABI Prism DNA sequencer. The sequences ob-

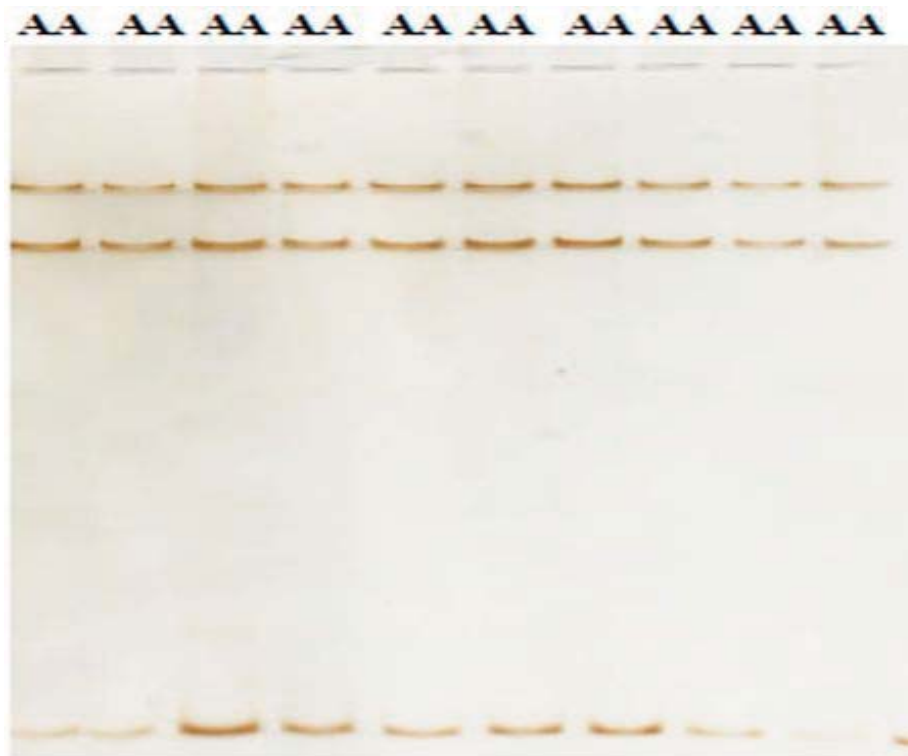


Fig. 2. PCR-SSCP genotypes of 269 bp fragment of CXCR2 gene

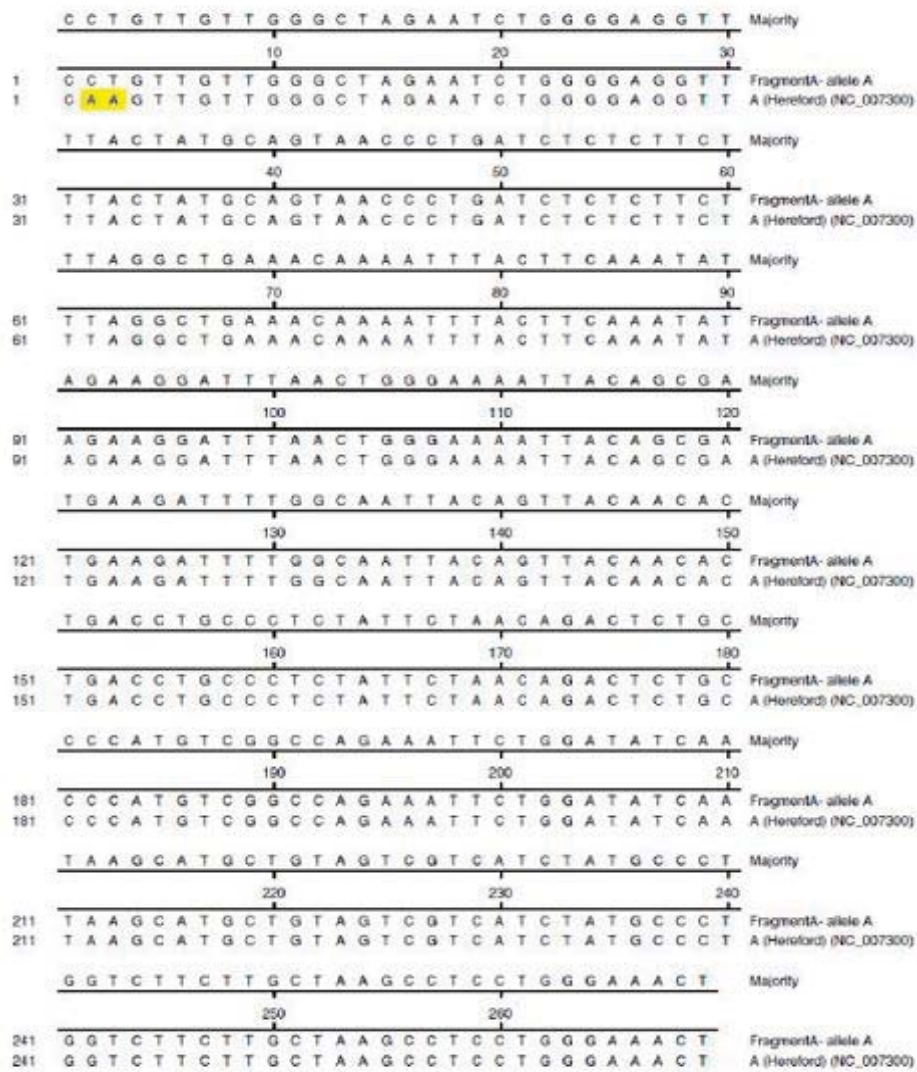


Fig. 3. Nucleotide sequence alignment 269bp fragment of CXCR2 receptor gene

tained were subjected to NCBI BLAST. After comparing with other available sequences of cattle, the amplified fragment was confirmed to comprise of the partial exonic region of CXCR2 gene. Sequences of Vrindavani and corresponding sequence of Hereford cattle were aligned using MegAlign programme of DNASTAR software (Fig. 3). From the alignment of the allelic variants, it was found that there are differences at two positions between Vrindavani cattle and Hereford cattle. Vrindavani has Cytosine and Thiamine at 2nd and 3rd positions respectively. After analyzing the sequences of 269 bp fragment of CXCR2 gene of Vrindavani cattle and Hereford cattle, it was found that there were variations at two positions (2nd and 3rd) in the intronic regions of CXCR2 receptor gene.

Discussion

Single nucleotide polymorphisms located within other regions of the bovine CXCR2 gene may af-

fect CXCR2 function during mastitis infections (Youngerman *et al.*, 2004). Several single nucleotide polymorphisms have been identified within different regions of the human CXCR2 gene (Kato *et al.*, 2000, Renzoni *et al.*, 2000). Other non-synonymous SNP located within segments of the CXCR2 coding sequence may affect receptor binding and function. Moreover, synonymous or non-synonymous SNP in the promoter and 3' untranslated regions may affect initiation or/and termination of transcription/translation. To determine whether other SNP are involved with changes in susceptibility or resistance, the complete sequence of the bovine CXCR2 gene should be determined.

Conclusion

The partial fragment of CXCR2 gene was found to be monomorphic in Vrindavani crossbred cattle.

The presence or absence of particular genotype can be used as a marker for breed identification. Other regions of this gene need to be sequenced for the complete characterization of this gene.

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