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RESEARCH ARTICLE
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Sequencing and phylogenetic analysis of partial *CXCR2* gene of Murrah buffalo

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Abstract

Aim: Present study was carried out to sequence and phylogenetic analysis of *CXCR2* gene of Murrah buffalo.

Materials and Methods: For the present investigation, from a group of forty eight Murrah buffaloes (*Bubalus bubalis*), blood samples were collected randomly from eight animals, out of which four were healthy and four were mastitic.

Results: The amplification of *Interleukin-8B (IL-8B)* receptor gene target sequence was carried out using the primer pair in an optimized polymerase chain reaction. Partial sequencing of *IL-8B* receptor gene of *Bubalus bubalis (*Murrah) has been done successfully. The sequences of *IL-8B* receptor gene showed 99% homology to that of *Bos indicus* × *Bos taurus*, 98% to that of *Bos taurus*, 97% to that of *Ovis aries*, 93% to that of *Sus scrofa*, 92% to that of *Equus caballus* and 90% to that of *Felis catus.*

Conclusion: From the present study it can be concluded that the PCR amplification procedure for target region of *IL-8B* receptor gene yielding 459 bp products has been standardized, which yielded consistent and specific amplification. Amplification of partial *IL-8B* receptor gene (exon 2- 459 bp) using self designed primers specific for cattle ortholog sequence signifies that the locus is conserved in cattle and buffaloes. In phylogenetic tree, the target sequence of *IL-8B* receptor gene of *Bubalus bubalis* were found to be more closely related to *Bos indicus* × *Bos Taurus and Bos taurus* than to *Ovis aries* and *Sus scrofa.*

Keywords: *CXCR2* gene, Murrah buffalo, phylogenetic analysis.

Introduction

Mastitis, an inflammatory reaction of mammary gland is the most dreaded disease for dairy farmers because of reduced milk production, increased treatment costs, labour, milk discarding following treatment, death and premature culling [1]. As resistance to mastitis is a polygenic trait, genes associated with neutrophil function are candidate genes for mastitis resistance. Neutrophils are the predominant cell type found in the mammary tissue/secretions during early inflammation and constitute more than 90% of the total leukocytes [2]. Several candidate pathways were also found in the study on integrated genomic data from genome-wide association mapping in cattle, and transcriptomic data from microarray studies on mastitis pathogens. Of great interest are *IL-17* and *IL-8* signaling pathways [3]. *Interleukin-8* (*IL-8*) is the main chemo- attractant in this process and binds on two receptors, namely *IL-8* RA (*CXCR1*) and *IL-8 RB* (*CXCR2*) present on neutrophils. *IL-8B* receptor is essential for neutrophil migration to the mammary gland and infection resolution. *CXCR2* binds to *IL-8* and oncogene α, neutrophil activating peptide-2. Earlier it was given the name CDw128b, but now it is changed to CD182. Chemokine receptors are G protein-coupled receptors containing 7 transmembrane domains that are found on the surface of leukocytes [4]. The *CXCR1* and *CXCR2* genes encoding receptors lay on the second chromosome approximately 20 kb separated from each other and on opposite strands. The gene located more centromeric encodes for *CXCR1*, the gene located more telomeric encodes for *CXCR2* [5]. Activation of *CXCR1* is believed to result in a wider array of antimicrobial processes than activation of *CXCR2*, but *CXCR2* might respond to a lower concentration of ligand [6]. The *CXCR2* gene in bovines is 7.5 kb in size consisting of two exons of 83 bp and 1089 bp separated by an intronic sequence length of 6328 bp. It gives a transcript length of 1172 bp and translation length of 363 amino acids [7]. Bovine IL-8 receptor loci (*CXCRl* and *CXCR2*) have been mapped approximately 90.3 cM from the centromere of bovine chromosome (BTA)2. The chemotactic cytokine belong to the chemokine superfamily which can be divided into four families (CXC, CX3C, CC and C) according to the position of the first two closely paired and highly conserved cysteines near the amino terminus of the protein [8]. The two N-terminal cysteine of CXC chemokines (α-chemokines) are separated by one amino acid, represented in this name with an "X".

There have been 17 different CXC chemokines described in mammals. CXC chemokine can be further classified into ELR-positive and ELR-negative molecule based on the presence or absence of a specific amino acid motif of glutamic acid –leucine-arginine (ELR for short) immediately before the first cysteine ELR-positive CXC chemokines specifically induce the migration of neutrophils, and interact with chemokine receptors *CXCR1* and *CXCR2*. An example of an ELR-positive CXC chemokine is interleukin-8, which induces neutrophils to leave the bloodstream and enter into the surrounding tissue [9]. *CXCR2* binds with *CXCL8* (*IL-8*) with high affinity, in addition to IL8, it also binds with different CXC ELR+ chemokines, including *CXCL1* (GRO-α), *CXCL2* (GRO-β), *CXCL3* (GRO-γ), *CXCL5* (epithelial cell-derived neutrophil-activating peptide-78), and *CXCL7* [10].

IL-8 is the most extensively studied chemokine to date. Other CXC chemokines that lack the ELR motif, such as *CXCL13*, tend to be chemoattractant for lymphocytes [11]. Intracellular signaling by chemokine receptors is dependent on neighbouring G-proteins. G-proteins exist as a heterotrimer and they are composed of three distinct subunits (α, β and γ). When the molecule GDP is bound to the G-protein subunit, the G-protein is in an inactive state. Following binding of the chemokine ligand, chemokine receptors associate with G-proteins, allowing the exchange of GDP for another molecule called GTP, and the dissociation of the different G protein subunits. The subunit called Gβ activates an enzyme known as Phospholipase C (PLC) that is associated with the cell membrane. PLC cleaves Phosphatidylinositol (4, 5)-bisphosphate (PIP2) to form two second messenger molecules called inositol triphosphate (IP3) and diacylglycerol (DAG). DAG activates another enzyme called protein kinase C (PKC), and IP3 triggers the release of calcium from intracellular stores. These events promote many signalling cascades, affecting a cellular response. *CXCL8* (*IL-8*) binds to its specific receptors, *CXCR1* or *CXCR2*, a rise in intracellular calcium activates the enzyme phospholipase D (PLD) that goes on to initiate an intracellular signaling cascade called the MAP kinase pathway. At the same time the G-protein subunit Gα directly activates an enzyme called protein tyrosine kinase (PTK), which phosphorylates serine and threonine residues in the tail of the chemokine receptor, causing its desensitisation or inactivation. The initiated MAP kinase pathway activates specific cellular mechanisms involved in chemotaxis, degranulation, release of superoxide anions, and changes in the avidity of cell adhesion molecules called integrins. Like many other chemoattractants, *IL-8* induces re-arrangement of the cytoskeleton, changes in intracellular Ca++ levels, activation of integrins, exocytosis of granule proteins, and respiratory burst [12, 13].

Therefore, the present study was carried out to sequence and phylogenetic analysis of *CXCR2* gene of Murrah buffalo.

Materials and Methods

Ethical approval: Approval for collecting blood samples was granted by Institutional Animal Ethics Committee.

Climatic Condition and Experimental Animals: Geographically, Hisar Haryana is located at 29.09°N 75.43°E in western Haryana, at an altitude of 215 above mean sea level. Hisar has a continental climate, with very hot summers and relatively cool winters. Summer season goes up to 46° C while winter goes down up to 1.5° C. For the present investigation, blood samples were collected from forty eight unrelated Murrah buffaloes *(Bubalus bubalis)* from Government Livestock Farm, Hisar. Approximately 10 ml of venous blood sample was collected randomly from eight animals in 15 ml sterile polypropylene centrifuge tube containing 0.5 ml of 0.5M EDTA as anticoagulant. Genomic DNA was isolated from blood samples as described by Sambrook and Russel method with minor modifications. The quality of genomic DNA was determined by agarose gel electrophoresis on 0.7 % w/v agarose. The purity of DNA was evaluated by taking the ratio of optical densities (OD) at 260 nm to that of 280 nm, by spectrophotometer (Biorad Smart SpecTM Plus). The samples having OD ratio between 1.7-1.9 were considered having acceptable purity and used in future experiments.

Partial exon 2 of *IL- 8B* gene was amplified by using the self-designed primers using FAST PCR software. Forward 5'..GACTCTGCCCCAT GTC GG .3'Reverse5′..GATGGCCCTGCGGAAGATG..'3. The reaction mixture for PCR was prepared in 0.2 ml thin walled PCR tubes. The master mix was prepared by adding 16.7 µl of nuclease free water (NFW), 2.5 µl of *DreamTaq*™ DNA polymerase buffer, 0.5 µl (100 µM) dNTPs, 1.0 µl of each primers (20 µM), 0.3 µl of Dream*Taq*™ DNA polymerase (Fermentas) and for each sample and after proper mixing it was distributed to all the tubes (22 µl to each tube). 3 µl of genomic DNA (50 ng) was added in all the tubes. All these steps were carried out on wet ice. The 25 µl reaction mix was kept for amplification in programmed thermocycler (Bio-Rad i-Cycler) with initial denaturation at 95°C for 5 min, then 35 cycles of denaturation at 95°C for 30 seconds (s), annealing at 64°C for 30 s and extension at 72°C for 1 min, the cycles were followed by a final extension at 72°C for 10 min. Amplification products were resolved by gel electrophoresis on 1.5 % agarose gel in TAE buffer, stained with ethidium bromide (1 µg/ml). 50bp ladder was used as molecular weight marker. PCR products were visualized by UV- trans-illuminator and photographed. PCR products were purified using QIAquick gel extraction kit (QIAGEN) to remove primer dimers and other PCR ingredients, before custom sequencing. Sequencing was done by using automated DNA sequencer Applied Biosystem 3130 XL Genetic Analyzer at Department of Animal Biotechnology, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Science, Hisar, Haryana, India. The vector specific

Table-1: Identity of *IL-8B* receptor gene (target region) of *Bubalus bubalis* with other Species

Accession number	*IL-8B* receptor gene of specific species	Query coverage (%)	Identity (%)
JF927834.1	*Bos indicus* x *Bos taurus*	98	99
JF927835.1	*Bos indicus* x *Bos taurus*	98	98
DQ328664.1	*Bos taurus*	98	98
XM004005420.1	*Ovis aries*	98	97
AK230995.1	*Sus scrofa*	98	93
XM003365149.1	*Equus caballus*	99	92
XM003991133.1	*Felis cattus*	99	90

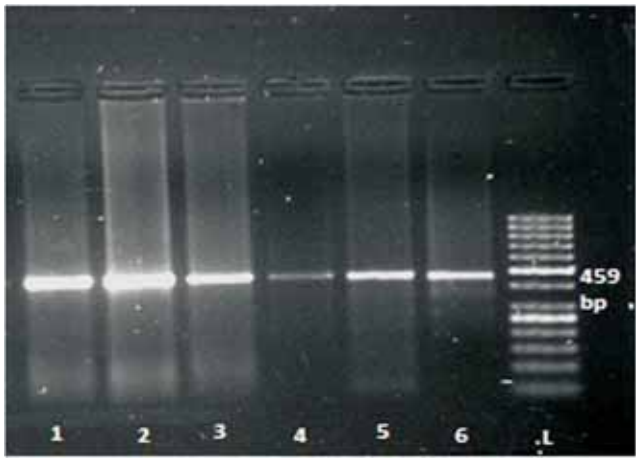

Figure-1: PCR amplification of *CXCR2* gene
Lane L: 50 bp ladder, Lane 1 to 6: PCR product of *CXCR2* gene (459 bp)

primer (Pjet1.2 Forward/ Pjet1.2 Reverse) were used for sequencing by BigDye® Terminator v 3.1Cycle Sequencing Kit (ABI). The reaction mixture for PCR was prepared in 0.2 ml thin walled PCR tubes. The master mix was prepared by adding 1.9 µl of nuclease free water (NFW), 2.0 µl of Dilution Buffer (5x), 0.6 µl DMSO as distributed to all the tubes (5.0 µl to each tube). Then added 2 µl of pJET1.2 Forward/Reverse sequencing primers (4 µM) in each tube. Then 3µl of plasmid was added in all the tubes. All these steps were carried out at 4°C. The 10 µl reaction mix was kept for amplification in programmable thermocycler (Bio-Rad i-Cycler) with initial denaturation at 96°C for 1 min then 25 cycles of denaturation at 96°C for 10 seconds (s), annealing at 50°C for 5 s and primer extension at 60°C for 4 min. After the final extension step, the PCR products were purified by BigDye Terminator v3.1Clean up (Tube Method). The sequences obtained from automated DNA sequencer were analyzed using NCBI BLASTn online software tool available on internet after converting sequences into FASTA format. ClustalW2 programme was used for multiple sequence alignment of buffalo *IL-8B* receptor gene sequence with other species DNA sequences retrieved from NCBI database. The phylogenetic tree was also constructed using MEGA4 software to show relatedness among the species with respect to *IL-8B* receptor gene fragment.

Results and Discussion

The amplification of *IL-8B* receptor gene target sequence was carried out using the primer pair in an optimized PCR. The molecular size of the PCR products was estimated to be 459 bp by comparing with DNA size markers (Figure-1). The sequence data generated after sequencing was compared with the sequences available in NCBI database and the maximum homology search was done using BLASTn. The sequences of *IL-8B* receptor gene showed 99% homology to that of *Bos indicus* × *Bos taurus* , 98% to that of *Bos taurus*, 97% to that of *Ovis aries*, 93% to that of *Sus scrofa*, 92% to that of *Equus caballus* and 90% to that of *Felis cattus* (Table-1). Shivanand *et al*. studied PCR-SSCP and sequencing of a 269 bp fragment of *CXCR2* receptor gene in Vrindavani cattle [14]. However no information is available about sequencing of bubaline *CXCR2* gene. Bovine homologs were identified to six human *CXCR* receptors (*CXCR1-6*) by bioinformatic analyses of available bovine sequences and genome databases [15]. Multiple sequence alignment of partial *IL-8B* receptor gene sequence of *Bubalus bubalis* was carried out among other species to study sequence based diversity globally. The alignment was carried out using ClustalW2 and Bioedit software. The alignment revealed highest similarity to *Bos indicus* × *Bos taurus* (Figure-2). The phylogenetic tree was constructed using MEGA4 software [16] to show relatedness between the species with respect to *IL-8B* receptor gene fragment (Figure-3). In phylogenetic tree, sequences of target region of *IL-8B* receptor gene of *Bubalus bubalis* are found more closely related to *Bos indicus* × *Bos taurus* than *Bos taurus, Ovis aries* and *Sus scrofa. Equus caballus* and *Felis catus* are the farthest species. The chemokine receptors are thought to have originated from a single ancestral gene with

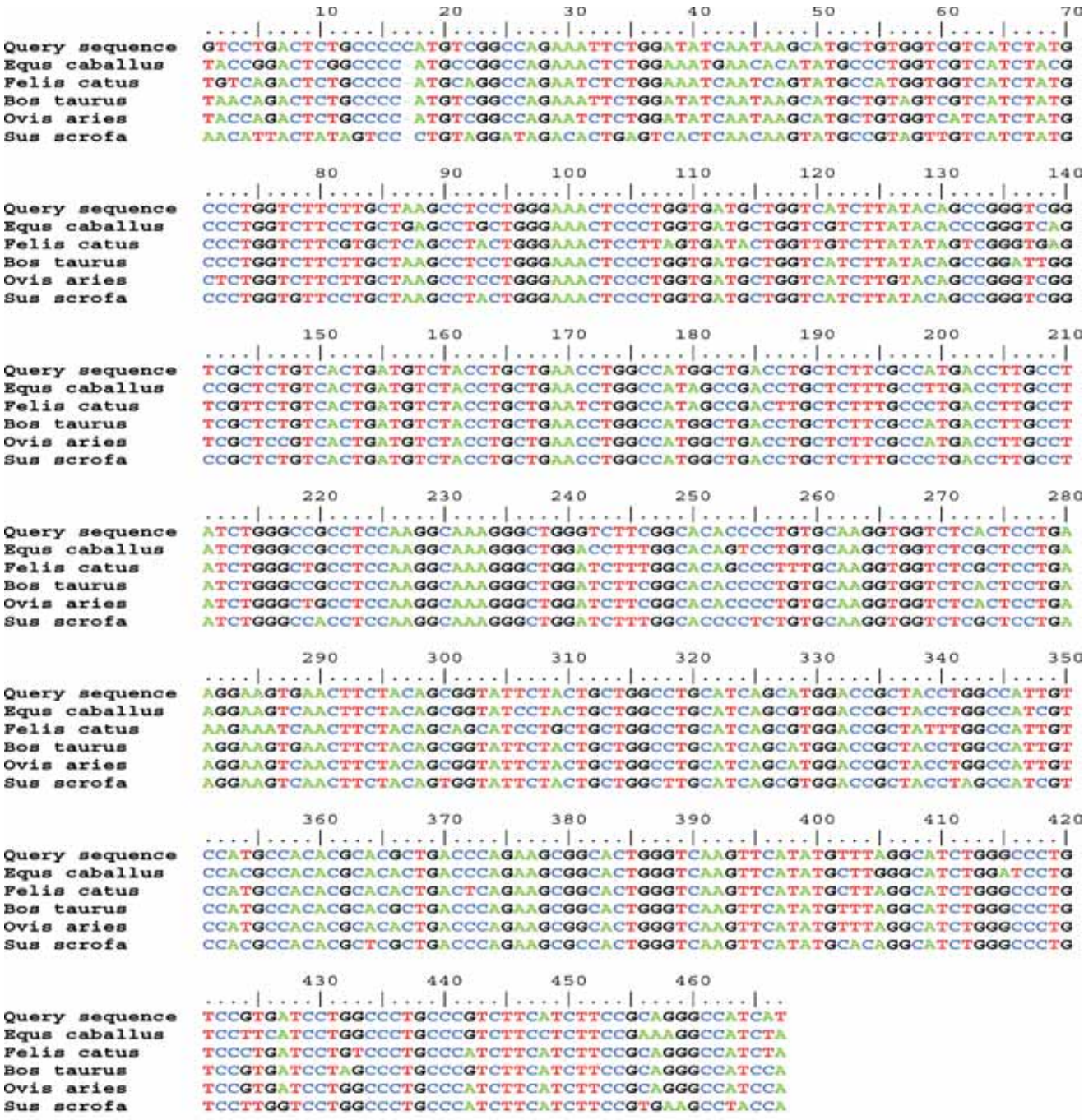

Figure-2: Multiple sequence alignment of *CXCR2* gene with different species.

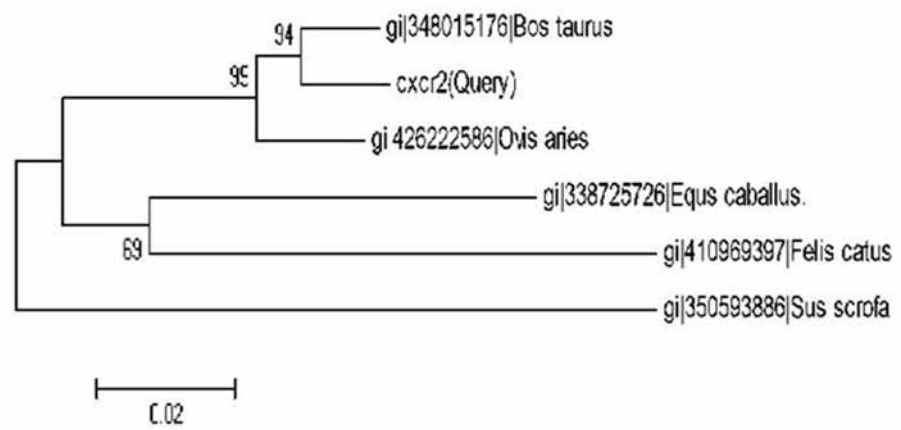

Figure-3: Phylogenetic tree of *CXCR2* gene region depicting the evolutionary relationship among different species.

individual genes arising due to subsequent gene duplication events [17]. Many of the chemokine receptors are clustered across the genome and these proximal chemokine receptors also tend to be closely related. Generally the chemokine receptor family is well conserved between mammalian species, as can be seen by a comparison of the well-studied human and murine chemokine receptor genes; however some differences do exist, indicating ongoing evolution of the chemokine receptor system.

Conclusion

From the present study it can be concluded that the PCR amplification procedure for target region of *IL-8B* receptor gene yielding 459 bp products has been standardized, which yielded consistent and specific amplification. Amplification of partial *IL-8B* receptor gene (exon 2- 459 bp) using self designed primers specific for cattle ortholog sequence signifies that the locus is conserved in cattle and buffaloes. Partial sequencing of IL-8B receptor gene of *Bubalus bubalis* (Murrah) has been done successfully. Basic Local Alignment Search Tool (BLAST) analysis revealed sequence identity of target region (*IL-8B* receptor gene) of Murrah buffalo with, 99% with *Bos indicus* × *Bos taurus* (JF927834.1), 98% with *Bos taurus* (DQ 328664.1), 97% with *Ovis aries* (XM004005420.1) and 93% with *Sus scrofa* (AK230995.1). In phylogenetic tree, the target sequence of *IL-8B* receptor gene of *Bubalus bubalis* are found more closely related to *Bos indicus* × *Bos Taurus and Bos taurus* than to *Ovis aries* and *Sus scrofa.* Molecular characterisation and phylogenetic studies of *CXCR2* gene can serve as guide for polymorphism and association studies with mastitis resistance for marker assisted selection breeding programme. Further this study can be used to identify cytokine markers which can be used to develop anti mastitis vaccines.

Authors' contributions

SAW planned and carried out research work under his MVSc thesis programme in collaboration with advisory members and guide MLS. MAD, AK, MAR and DB played a helping hand role in carrying of experiment and computational analysis of data. All authors read the manuscript and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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