INTRODUCTION

Toll-like receptors (TLRs) play an important role in the recognition of invading pathogens and the modulation of innate immune responses in mammals. The TLR4 and TLR7 are well known to recognize the bacterial lipopolysaccharide (LPS) and single-stranded (ssRNA) ligands, respectively, and play important role in host defense against Gram-negative bacteria and ssRNA viruses. In the present study, coding exon fragments of these two TLRs were identified, cloned, sequenced and analyzed in terms of insertion-deletion polymorphism, within bovine TLRs 4 and 7, thereby facilitating future TLR signaling and association studies relevant to bovine innate immunity. Comparative sequence analysis of TLR 4 exons revealed that this gene is more variable, particularly the coding frame (E3P1), while other parts showed percent identity of 95.7% to 100% at nucleotide and amino acid level, respectively with other Bos indicus and Bos taurus breeds from different parts of the world. In comparison to TLR4, sequence analysis of TLR7 showed more conservation among different B. indicus and B. taurus breeds, except single point mutation at 324 nucleotide position (AAA to AAM) altering a single amino acid at 108 position (K to X). Percent identity of TLR7 sequences (all 3 exons) was between 99.2% to 100% at nucleotide and amino acid level, when compared with available sequence database of B. indicus and B. taurus. Simple Modular Architecture Research Tool (SMART) analysis showed variations in the exon fragments located in the Leucine Rich Repeat (LRR) region, which is responsible for binding with the microbial associated molecular patterns and further, downstream signaling to initiate anti-microbial response. Considering importance of TLR polymorphism in terms of innate immunity, further research is warranted. (Key Words : Bovine TLR 4, TLR 7, Exons, LRR, Innate Immunity, Sequence Analyses)
signaling molecules and initiates innate cellular immune responses (Muzio et al., 2000). TLRs comprising of Lucien rich repeat (LRR) and TIR domain, are responsible for recognizing the microbial ligand and downstream signaling, respectively. The intracellular TIR domain is highly conserved with functional similarity among species and TLR genes, as it is involved in engaging signaling pathways within cells (Beutler and Rehli, 2002). However, the extracellular TLR domains exhibit significantly-higher divergence reflecting their involvement in MAMP recognition from multiple microbial sources (Zhou et al., 2007). TLR4 recognizing bacterial cell components are critical in the immune response against Gram positive and negative bacteria (Underhill et al., 1999). On another side, upon viral infection, TLR7 recognizes ssRNA viral nucleic acid released in intracellular acidic compartments of phagocytes, which take up virus infected cells and mount appropriate anti-viral innate immune response by inducing type I interferons (Diebold et al., 2004).

Despite growing interest in the investigation of TLR involvement in host defense against microbial infections of human and murine, nothing on TLR patterns of bovine has been reported, as of yet. Perusal of literature shows that limited information is available on TLRs of Indian cattle, in particular on cattle from high altitude areas. Thus the objective of this study was to characterize the TLR 4 and 7 particularly on cattle from high altitude areas. Thus the limited information is available on TLR patterns of bovine has been reported, as of yet. Perusal of literature shows that limited information is available on TLR patterns of bovine.

TLR4 recognizing bacterial cell components are critical in the immune response against Gram positive and negative bacteria (Underhill et al., 1999). On another side, upon viral infection, TLR7 recognizes ssRNA viral nucleic acid released in intracellular acidic compartments of phagocytes, which take up virus infected cells and mount appropriate anti-viral innate immune response by inducing type I interferons (Diebold et al., 2004).

### MATERIALS AND METHODS

**Isolation and stimulation of peripheral blood mononuclear cells (PBMCs)**

Blood samples used in this study were obtained from three healthy non-descript hill cattle of Kumaon region of Uttarakhand, India. The region lies in temperate western Himalaya with an altitude of more than 2,300 meters. The PBMCs were retrieved from 5 ml heparinized whole blood overlaying Ficoll (Sigma-Aldrich, St. Louis, USA) by density gradient centrifugation at 1,900 rpm for 45 min. The PBMCs obtained from the buffy coat were counted in haemocytometer and their viability was determined by Trypan Blue staining. PBMCs were plated in 6 well tissue culture plate (Nunc, Germany) with cell concentration of 1x10⁶ per ml with RPMI-1640 (Sigma-Aldrich, St. Louis, MO) enriched with 10% FBS (Hyclone, USA) and stimulated with Concanavalin A (ConA) (Sigma-Aldrich, St. Louis, MO) at the concentration of 5 µg/µl per wells except the control well. Samples were harvested at the 3 h interval.

**Genomic DNA isolation**

The total genomic DNA was isolated from the PBMCs using the QIAamp DNA Mini kit (Qiagen, Germany) following the manufacturer’s instructions. DNA yield and quantity were assessed by Nanodrop Spectrophotometer (ND-1000, Thermo Scientific, USA). The genomic DNA (gDNA) was stored in 1X TE buffer (0.25 M EDTA sodium salt, 0.25 M TRIS-HCl, pH 8.0) (Sigma-Aldrich) at -20°C till further use.

**Amplification and cloning of TLR4 and 7 exons**

The exons were selected and primers were designed from the published sequence available on NCBI database of TLR4 gene of *Bos taurus* (Acc no. DQ839567) and TLR7 gene of *Bos indicus* (Acc no. EF076738) using GeneTool Lite 1.0 software (BioTools Inc., Edmonton, Canada). The primers were designed to cover the LRR region of both the genes (TLR 4 and 7), which is mainly responsible for recognizing the pathogen associated conserved motifs. The primer pairs used in the study are listed in Table 1. PCR conditions were optimized and carried out with 100-200 ng of the gDNA as a template with 2.5 µl 10× PCR buffer, 1.5 µl 25 mM MgCl₂, 1 µl 10 mM dNTPs, 1 µl of 10 pmole each of forward and reverse primers, 1 µl of Taq DNA polymerase, LC (recombinant) (Fermentas, Lithuania) and the volume was made up to 25 µl using Nuclease free water. The PCR was carried out with a initial denaturation at 94°C for 8 min, followed by 30 cycles with denaturation at 94°C for 45 s, annealing at specific Tm for the primer pairs (as per exon fragment) for 30 s and extension at 72°C for 1 min.

### Table 1. List of primers used for the amplification of Toll like receptors 4 and 7 exons with predicted size amplicons

<table>
<thead>
<tr>
<th>TLRs</th>
<th>Exons</th>
<th>Forward</th>
<th>Reverse</th>
<th>Sequences 5'-3'</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4</td>
<td>E3-P1</td>
<td>TTTGCCGGAATTTCTCCTTGTCTATCTG</td>
<td>AGTGCCCTACGTTGCGGAGTTAG</td>
<td>444</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E3-P2</td>
<td>TTCGGCATTGGACATCTCAAAAACCTT</td>
<td>TATCCGGAAATGTCTAATTGCGAGTTAG</td>
<td>441</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E3-P3</td>
<td>TGGGGAATTTAAAATATGAAAGGAA</td>
<td>CTAAGCCCATGGAAGTTTAGACCTAAG</td>
<td>487</td>
<td></td>
</tr>
<tr>
<td>TLR7</td>
<td>CDS-P3</td>
<td>AGTGGGAAATTCGCGCTTGT</td>
<td>CAGCTCTTGCCATACTCATCA</td>
<td>635</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CDS-P5</td>
<td>CAGCAGGACCATGGAGGTGAAATC</td>
<td>CATCGTGGCGCCAAAAGTAAGGATAGTC</td>
<td>574</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CDS-P7</td>
<td>AGGTGCTTTCCAGTTGCGACATCT</td>
<td>TGCCACCCTTCTCCACAGTATT</td>
<td>1,186</td>
<td></td>
</tr>
</tbody>
</table>
The final extension was performed for 10 min at 72°C. The amplicons were checked for their expected sizes on 1% agarose gel. Expected exon amplicons of TLR 4 and TLR 7 were excised from the gel and purified using GeneJET gel extraction kit (Fermentas). The purified products were cloned into pGEMT-Easy vector (Promega, Madison, USA). The ligation reaction was performed at 22°C for 3 h. The recombinant plasmids were transformed into E. coli Top10 competent cells (Invitrogen, USA) as per standard protocols. Four white colonies were screened for the presence of the insert by colony PCR and release of the inserts from the recombinant plasmid by Restriction Enzyme digestion (Fermentas). The positive clones were identified with extraction of plasmid by using GeneJET Plasmid Miniprep kit (Fermentas).

**Sequencing and bioinformatics analysis**

The insert-positive plasmids were sequenced by an Automated DNA Sequencer (Applied Biosystems 3130 Genetic Analyzer) and the sequences of cloned TLR 4 and 7 exons were analysed using the LaserGene software package (DNASTAR Inc., Madison, WI). MegaBlast was used to identify mammalian TLR nucleotide sequences within the non-redundant nucleotide database (http://www.ncbi.nlm.nih.gov/) by comparison with bovine TLR sequences. The alignment of coding sequences of TLR4 and 7 exons from multiple species was done using the program Clustal W. The Simple Modular Architecture Research Tool (SMART) was employed to predict the domain structure of B. indicus (Sahiwal) TLR 4 and 7 (http://smart.embl-heidelberg.de/) (Schultz et al., 1998). To analyze sites located within the LRR solenoid structure for B. indicus from Kumaon region TLR4 and TLR7, individual LRRs were aligned manually, based on the conserved motif, xLxxLxLxxNxL, in which L is Leu, Ile, Val, or Phe and N is Asn, Thr, Ser, or Cys (Weber et al., 2004; Matsushima et al., 2007). Annotation of manually aligned LRR sequences was compared to the domain structure predicted by SMART to assign transmembrane and TIR domains.

Sequence data from these samples were entered in the NCBI GenBank database under the following accession numbers: TLR7 exon fragments-CDS3P (HM363557), CDS P5 (HM363558), CDS7P (GU143097) and TLR4 exon fragments- E3P1 (HM363554), E3P2 (HM363555) and E3P3 (HM363556).

**RESULTS AND DISCUSSION**

Since the discovery of the Toll gene for the establishment of dorso-ventral polarity in the developing embryo in Drosophila (Hashimoto et al., 1988) and its antifungal function in adult flies (Lemaître et al., 1996), many TLRs have been cloned in mammals and shown to play a critical role in linking innate and adaptive immunity (Janeway and Medzhitov, 2002; Takeda et al., 2003; Akira and Takeda, 2004; Pasare and Medzhitov, 2004). We report that TLR4 and TLR7 of high altitude adapted B. indicus from sub-Himalayan region are highly homologous to other B. indicus and B. taurus breeds from tropical, sub-tropical and semi-arid regions of India and other countries and also have conserved TIR domain with variations in LRR motifs.

In the present study, 80% of the bovine PBMCs obtained through density gradient centrifugation were viable after Trypan blue staining. Blastogenesist of bovine PBMCs was observed on ConA stimulation, which was not observable in the control wells. The expected PCR amplicons of 444 bp (E3P1), 441 bp (E3-P2) and 487 bp (E3-P3) for TLR4 exons (Figure 1) and specific amplicons of 635 bp (CDS-P3), 574 bp (CDS-P5) and 1,186 bp (CDS-P7) for TLR 7 exons (Figure 2) were observed in 1% agarose gel after 30 cycles of PCR amplification. These all PCR products were also confirmed by sequencing and blast analyses.

Analysis of TLR4 exon sequences of B. indicus from Himalayan region showed more variations in comparison to the TLR7 sequences of cattle from this region. The initial exon region (E3P1) analysis showed that amino acid residues (1-38) are highly variable among B. indicus and B. taurus breeds, whereas amino acid residues from 39-132 are less variable at both nucleotide and amino acid level, except the Bubalis bubalis, which showed 96% identity at nucleotide level and 95.7% at amino acid with 9 substitutions in nucleotide with reflected changes in amino acid at 5 positions (Table 2). Other TLR 4 regions (E3P2 and E3P3) showed 96.6% and 97.5% identity at nucleotide level and 93.9% and 96.3% at amino acid level, respectively with B. indicus and B. taurus breeds from tropical, sub-tropical and semi-arid regions of India and other countries and also have conserved TIR domain with variations in LRR motifs.

The comparative nucleotide and deduced amino acid analyses of TLR7 exon sequences with cognate genes of other bovine species revealed that B. indicus from temperate sub-Himalayan region shows more than 99.2% to 99.8% sequence homology with other B. indicus breeds (Sahiwal, Hariana, Kankrej, Gir and Red Sindhi) from tropical, sub-tropical and semi-arid regions, while 100% conservation was seen with B. indicus (Brahman and Nelore) breeds and B. taurus (Braford, Angus and Holstein) at nucleotide and amino acid levels. Though the TLR7 sequences among B. indicus from Himalayan region and other B. indicus and B. taurus breeds were highly conserved, single point mutations were observed at 324 nucleotide position (AAA to AAM) with change in one amino acid at
Figure 1. Positions of TLR exons. (A) Schematic representation of Toll like receptor (TLR) 4 exons positions, (B) PCR amplification of TLR4 exons. Lanes indicate, M: 100 bp DNA ladder, 1: E3P1 (444 bp amplicon), 2: E3P2 (441 bp amplicon), and 3- E3P3 (487 bp amplicon) on 1% agarose gel.

Figure 2. Positions of TLR exons. (A) Schematic representation of TLR 7 exons, (B) PCR amplification of TLR 7 exon fragments. Lanes indicate, M: 100 bp DNA ladder, 1: CDS-P7 (1,186 bp amplicon), 2: CDS-P3 (635 bp amplicon), and 3: CDS-P5 (574 bp amplicon) on 1% agarose gel.
108 position (from K to X) in Sahiwal, Kankrej, Gir and Red Sindhi. Sequence analyses showed that TLR7 CDSP5 and CDSP7 exons showed 100% conservation among all breeds of *B. indicus* and *B. taurus*, except *B. indicus* Hariana breed in which 4-5 point mutations were noticed at nucleotide level with reflected amino acid changes of 2 amino acids (Table 3). SMART analyses comparison with Sahiwal showed that TLR7 CDSP3 was falling in the region of LRR and CDSP5 and 7 in the TIR domain.

Sequence variation at amino acid residues responsible for pattern recognition (MAMP-ligands) enables a faster adaptation to new pathogens, as they are encountered in different geographical locations or as they newly emerge in a habitat. This has clear selective advantages. Such advantageous substitutions spread faster in the populations than random substitutions. In the present study also changes appeared to be concentrated in the extracellular region of TLR4, particularly in the E3P1 region of LRRs. Changes like this, responsible for ligand binding have recently been reported (Jin et al., 2007). Differences between species in ligand recognition are known for several TLRs (Poltorak et al., 2000; Keestra et al., 2008) and can result in species-specific immune responses to certain pathogens. The relative genetic disparity between *B. indicus* and *B. taurus* is well established (Loftus et al., 1994). The genetic relatedness between *B. indicus* from temperate region explains that some of the differences seen in TLR4 are not just by random events over the time they diverged, but also by different microbial environments that the animals evolve within. The *B. indicus* breed analysed herein originated in temperate western Himalayan climate where the pathogen population is generally different to that encountered by the *B. indicus* or *B. taurus* breeds from tropical, semi-tropical or semi-arid areas. Consequently, there will be different selective pressures acting on the TLRs and other immune related genes in these species, which would result in sites being differentially fixed in their TLR genes.

In mammals, members of the TLR gene family play a
primary role in the recognition of pathogen-associated molecular patterns from bacteria, viruses, protozoa and fungi. Single nucleotide polymorphisms (SNPs) within TLR genes in humans seem to be associated with susceptibility to infection by specific diseases (Pandey et al., 2006). In livestock sector also there is accumulating evidence that genetic variations in TLR genes might be associated with disease resistance or susceptibility. Within bovine TLR2, polymorphisms at amino acid positions 227, 305 and 326 mapped to functionally important sites of TLR2 and is found as candidate SNPs for immune related traits in cattle. Nucleotide sequences of bovine TLR2, TLR4 and TLR6 genes have been screened to identify novel SNPs that can be used in studies of cattle resistance to diseases. As sites can lose or gain functional importance during evolution and several studies have shown that mutations in the TLR may reduce the ability of the protein to recognize PAMP and hence interfere with innate immune activation. Since no phenotypes on disease resistance are currently available for hill cattle it will not be possible to link the TLR4 and 7 polymorphism noticed in present study with the traits. This study is a step forward to sequence characterize the TLR4 and TLR7 genes of relatively unexplored population of hill cattle of India. Still more studies are required to determine TLR4 and TLR7 functional effect on the immune response after stimulation with relevant ligands and/or their association with immune related traits in animals. Work on genetic variations in these TLRs in relation to resistance against specific diseases in livestock may be useful in guiding genetic selection for disease resistance. This would facilitate the identification of particular disease susceptibility/resistance in cattle and will provide a valuable tool for the breeding industry to improve genetic resistance against a range of pathogens.

**ACKNOWLEDGMENTS**

The work was carried out under an Indian Council of Agricultural Research (ICAR)-NAIP funded project. Authors wish to thank the Head cum SIC, IVRI Mukteswar and Director, Indian Veterinary Research Institute, Izatnagar (UP), India for extending the infrastructure facilities for this project.

**REFERENCES**


Akira, S., S. Uematsu and O. Takeuchi. 2006. Pathogen


