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Genomic Analyses of Toll-like Receptor 4 and 7 Exons of *Bos indicus* from Temperate Sub-himalayan Region of India

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ABSTRACT : 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, respectivley 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)

INTRODUCTION

Toll like receptors (TLRs), being members of the Pattern Recognition Receptor (PRR) play a vital role in stimulating the innate immune system and are integral part of the innate immune system (Aderem and Ulevitch, 2000; Medzhitov and Janeway, 2000; Takeda et al., 2003). TLRs have been detected in invertebrates, vertebrates and plants (Takeda et al., 2003). The term "Toll-like receptors" was proposed in 1997 for mammalian proteins structurally related to the "TOLL" cell surface receptor seen in the Drosophila larvae. The TLR detects invading pathogens through conserved molecular structures known as Microbe Associated Molecular Patterns (MAMPs), such as bacterial cell wall components and microbial nucleic acids (Akira and Takeda, 2004). TLRs also trigger the activation of adaptive immune system by promoting presentation of antigens and up-regulation of various cytokines and co-stimulatory molecules (Takeda et al., 2003; Akira and Takeda, 2004; O'Neill, 2004). To date, at least 11 TLR members have been described in mammals (Akira et al., 2006; West et al., 2006). Although TLR family proteins are well conserved, accumulating evidence has showed that different members of the TLR family have very distinct functions in host defense. The key characteristics that distinguish individual TLR are ligand specificity, signal transduction pathways and sub cellular localization (Barton et al., 2006).

As appropriate pathogen recognition is crucial for mounting the immune response that can effectively combat the invading organism, a comprehensive understanding of pathogen recognition is necessary. The TLRs consist of a large extracellular domain responsible for MAMP binding, a transmembrane domain and an intracellular Toll/interleukin-1 receptor (TIR) domain which binds

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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 which are receptors for bacterial lipopolysaccharide ligand (LPS) and ssRNA viruses in order to understand the role of TLRs in regulating immune response in bovine. Three exon fragments (E3-P1, E3-P2 and E3-P3) of TLR4 and 3 exons of TLR7 genes (CDS-P3, CDS-P5 and CDS-P7) representing LRR and TIR domains were amplified, cloned, sequenced and analyzed.

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 1×10^6 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 $MgCl_2$, 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

TLRs	Exons	Sequences 5'-3'		Amplicon size (bp)
		Forward	Reverse	
TLR4	E3-P1	TTTGCCGGACTTCTTTGTTTCATCTG	AGTGCTCCAGGTTGGGCAGGTTAG	444
	E3-P2	TTCCCCATTGGACATCTCAAAAACTT	TATCCGGAATTGTTCAATGGTCAGGT	441
	E3-P3	TTGGGAGAATTTAAAAATGAAAGGAA	CTAAGCCCATGAAGTTTGAACCTAAG	487
TLR7	CDS-P3	AGTGGAAATTGCCCTCGTT	CAGCTCCTTGCATACTCATCA	635
	CDS-P5	CAGCAGGACCATGGAGAGTGAATC	CATCTGTGGCCAAGTAAGGAATAGTC	574
	CDS-P7	AGGTGCTTTCCAGTTGCGACATCT	TGCCACCCTTCTCCCAACAGTATT	1,186

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 Anlayzer) 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, IIe, 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-CDSP3 (HM363557), CDS P5 (HM363558), CDSP7 (GU143097) and TLR4 exon fragments- E3P1 (HM363554), E3P2 (HM363555) and E3-P3 (HM363556).

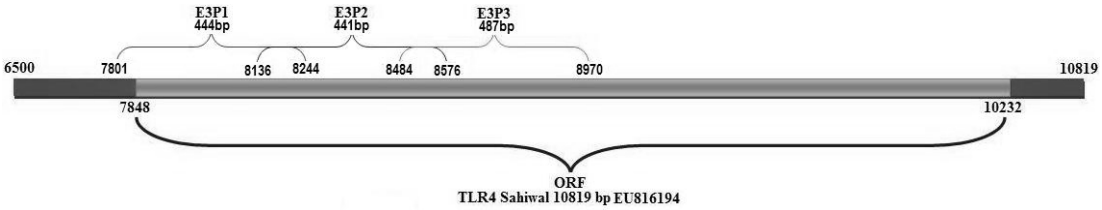
RESULTS AND DISCUSSION

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 (Lemaitre 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.

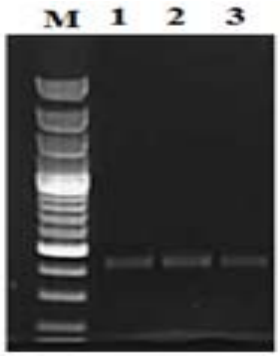
In the present study, 80% of the bovine PBMCs obtained through density gradient centrifugation were viable after Trypan blue staining. Blastogenesis of bovine PBMCs was observed on ConA stimulation, which was not observable in the control wells. The expected PCR amplicons of 444 bp (E3P1), 441 bp (E3-P2) 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. bubalis*. A total of 9-14 nucleotide substitutions with 5-6 amino acid variations were observed. However, other exons of TLR4 showed conservation of 99-100% at nucleotide and amino acid level with other *B. indicus* and *B. taurus* breeds (Table 2). SMART analyses showed that TLR4 E3P1, E3P2 are falling in the region of LRR domain.

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

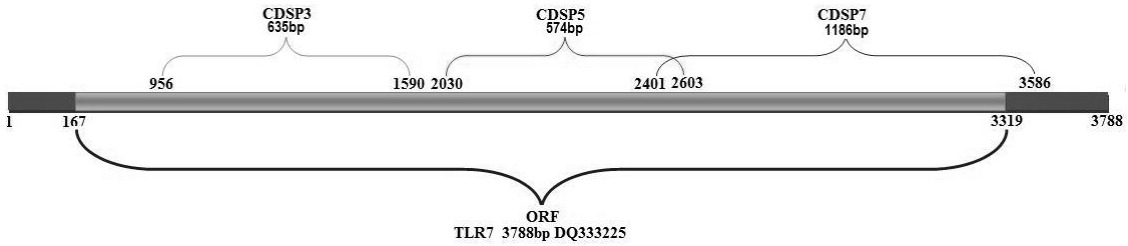


(A)

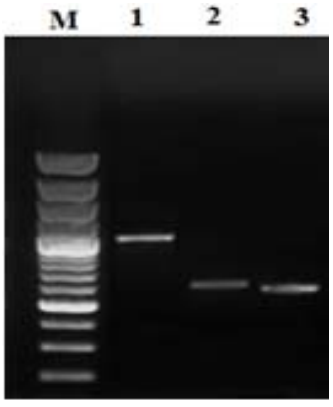


(B)

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% agarsoe gel.



(A)



(B)

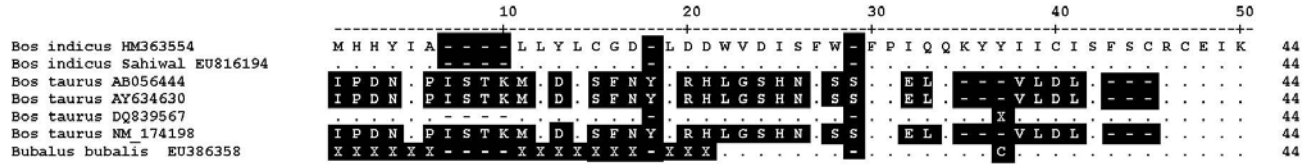
Figure 2. Positions of TLR exons. (A) Schematic representation of TLR 7 exons, (B) PCR amplification of TLR 7 exon fragments. Lanes indicate, M: 100 bp DNA ladder, 1: CDS-P7 (1,186 bp amplicon), 2: CDS-P3 (635 bp amplicon), and 3: CDS-P5 (574 bp amplicon) on 1% agrose gel.

Table 2. Comparison of TLR4 exon fragments (E3P1, E3P2 and E3P3) nucleotide and deduced amino acid sequences, where notable genetic differences were observed between *B. indicus* (Hill cattle) and other *B. indicus* and *B. taurus* breeds. Nucleotide changes are indicated in colored letter with altered position, and the E3P1 alignment data of deduce amino acid from 1-38 is provided in lower portion in dark lines

Species/ Accession number	TLR4_E3P1				TLR4_E3P2				TLR4_E3P3			
	NA*		AA**		NA		AA		NA		AA	
	% identity	Altered position	% identity	Altered position	% identity	Altered position	% identity	Altered position	% identity	Altered position	% identity	Altered position
Bos indicus Sahiwal EU816194	100	NC	100	NC	99.8	84 TTC/TTT	100	NC	99.6	152 GCT/GTT 342 CCG/CCA	99.4	51 A/V
Bos taurus AB056444	99.6	352 AAA/AGA	98.9	113 K/R	99.5	29 AAA/AGA 84 TTC/TTT	99.3	10 K/R	99.4	152 GCT/GTT 342 CCG/CCA 390 GGG/GGT	99.4	51 A/V
Bos taurus AY634630	100	NC	100	NC	99.8	84 TTC/TTT	100	NC	99.4	152 GCT/GTT 342 CCG/CCA 390 GGG/GGT	99.4	51 A/V
Bos taurus Chinese Simmental DQ839567	100	NC	100	NC	99.8	84 TTC/TTT	100	NC	99.4	152 GCT/GTT 342 CCG/CCA 390 GGG/GGT	99.4	51 A/V
Bos taurus Chinese Simmental NM_174198	100	NC	100	NC	99.8	84 TTC/TTT	100	NC	99.4	152 GCT/GTT 342 CCG/CCA 390 GGG/GGT	99.4	51 A/V
Bubalus bubalis EU386358	96	127 TAT/TGT 254 GGA/GGC 269 CTA/CTC 280 CAG/CGG 311 GTC/GTA 318 & 320 AAT/CAC 343 AAA/AGA 345 AAC/GCC	95.7	31 Y/C 82 Q/R 95 N/H 103 K/R 104 N/A	96.6	20 AAA/AGA 22 & 23 AAC/GCC 84 TTC/TTT 143 TAT/ TCT 145 TAT/ CAT 218 GAC/ GGC 290 TCA/TTA 335 ACC/ATC 380 CAA/AAA 383 AGA/ATA 387 TTT/TTC 435 TTC/TTT	93.9	7 R/K 8 A/N 48 Y/S 49 Y/H 73 D/G 97 S/L 112 T/I 127 Q/K 128 R/I	97.5	31 CAA/AAA 35 AGA/ATA 39 TTT/TTC 87 TTC/TTT 106 AAA/GAA 120 GAC/GAT 152 GCT/GTT 235 ATT/GTT 252 GAC/GAA 342 CCG/CCA 390 GGG/GGT 429 AAG/AAA	96.3	11 Q/K 12 R/I 36 K/E 51 A/V 79 I/V D/E

M = A/C, W= A/T, X = Fake amino acid, NC = No change.

* E3P1 alignment data from 153 to 432nt, ** E3P1 alignment data of deduce amino acid from 39 to 132.



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

Table 3. Nucleotide and deduced amino acid sequence comparison of Toll like receptor 7 exon fragments (CDS-P3, CDS-P5 and CDS-P7) where notable genetic differences were observed between *B. indicus* (Hill cattle**)** and other *B. indicus* and *B. taurus* breeds[1]

Species/Accession numbers	Toll like receptor 7											
	CDSP3				CDSP5				CDSP7			
	NA		AA		NA		AA		NA		AA	
	% identity	Altered position	% identity	Altered position	% identity	Altered position	% identity	Altered position	% identity	Altered position	% identity	Altered position
Bos indicus Brahaman EF076734	100	NC	100	NC	100	NC	100	NC	100	NC	100	NC
Bos indicus Sahiwal GU817062	99.8	324 AAA/AAM	99.5	108 K/X	100	NC	100	NC	100	NC	100	NC
Bos indicus Hariana GU046911	99.2	554 AGG/ATG 569 GAA/GCA 582 CCC/CCT 598 CTG/TTG	99.1	185 R/M 190 E/A	99.3	357 ACA/ACG 405 AGC/AGT 478 CTG/TTG 543 ACC/ACT	100	NC	99.5	34 GCT/GTT 107 TCT/TTT 172 CCG/CTG 296 TGT/TGC 342 ATA/ACA	99.3	99 F/L 114 I/T
Bos indicus Kankrej GU817059	99.8	324 AAA/AAM	99.5	108 K/X	100	NC	100	NC	100	NC	100	NC
Bos indicus Gir GU817058	99.8	324 AAA/AAM	99.5	108 K/X	100	NC	100	NC	100	NC	100	NC
Bos indicus Rathi GU817060	99.8	324 AAA/AAM	99.5	108 K/X	100	NC	100	NC	100	NC	100	NC
Bos indicus Red_Sindhi GU817061	99.8	324 AAA/AAM	99.5	108 K/X	100	NC	100	NC	100	NC	100	NC
Bos indicus Nelore EF076738	100	NC	100	NC	100	NC	100	NC	100	NC	100	NC
Bos taurus Holstein EF076736	100	NC	100	NC	100	NC	100	NC	100	NC	100	NC
Bos taurus Angus EF076732	100	NC	100	NC	100	NC	100	NC	100	NC	100	NC
Bos taurus Braford EF076733	100	NC	100	NC	100	NC	100	NC	100	NC	100	NC

[1] Altered nucleotide position is indicated in colored alphabet. M = A/C, W = A/T, X = Fake amino acid, NC = No change.

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.

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