Research in Veterinary Science 117 (2018) 144–149

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Distribution of single nucleotide polymorphisms and protein domain architecture of toll-like receptor-2 in Pahari cattle (Indian non-descript indigenous breed)

Verma Subhash[a,*], Sodhi Monika[c], Salwan Richa[a], Shekhar Chander[a], Singh Geetanjali[b], Sharma Mandeep[a]

[a] Department of Veterinary Microbiology, DGCN-COVAS, CSK HPKV, Palampur, HP 176 062, India
[b] Department of Veterinary Physiology and Biochemistry, DGCN-COVAS, Palampur, India
[c] Animal Biotechnology, NBAGR, Karnal, Haryana, India

ARTICLE INFO

Keywords:
Toll-like receptor-2
TLR
SNPs
Polymorphism
Indian zebu
Cattle
Bos taurus

ABSTRACT

Distribution of polymorphism in TLR2 gene was studied in Pahari (Indian non-descript indigenous breed) cattle of Himachal Pradesh. The complete sequence of TLR2 gene consisted of 136 bp 5'-UTR, 2355 bp CDS and 1316 bp 3'UTR. The phylogenetic analysis revealed clustering of Indian Zebu with *Bos taurus* as the nearest neighbour. The nucleotide sequences of TLR2 revealed the occurrence of 19 SNPs including 47% non-synonymous and 53% synonymous SNPs within TLR domain among all bovines. The dS/dN ratio (ω) < 1 at polymorphic sites revealed purifying selection and thus, indicating the presence of highly conserved domains. Two SNPs in the extracellular domain of TLR2 were predicted to have damaging effect whereas the rest have benign effect. The amino acid sequence of the coding region corresponds to a protein of 784 amino acids long with predicted molecular weight 104 kDa and 6.97 pI value. The amino acid sequence analysis showed extracellular domain composed of leucine rich repeats (LRR), trans- membrane domain and Toll-IL receptor domain in the protein. The 3D structure of TLR2 is a solenoid-like having active sites buried in the concave side and forming a pocket for ligand binding. The substitution of G with T in nucleotide sequence leads to the replacement of amino acid W ↔ L at position 119 which has not been reported so far. These findings indicate that polymorphism may be associated with PAMPs mediated differential TLR signaling of bovine immunity and could determine the outcome of infection.

1. Introduction

Toll-like receptors (TLRs) are evolutionary conserved proteins and functions in host defense processes against pathogenic infections (Janssens and Beyaert, 2003). TLRs are found in both vertebrates and invertebrates and belong to pattern recognition receptor (PRR) molecules (Takeda et al., 2003). TLRs are distinguished by the presence of Toll/IL-1 receptor (TIR) domain and LRRs in the extracellular domain for the recognition of bacterial, viral, protozoan and fungal PAMPs (Vasselon et al., 2002, Kaisho and Akira, 2006; Mukherjee et al., 2016). TLR2 plays important role in innate immunity and respond to gram positive pathogens associated molecular patterns (PAMPs) (Tantia et al., 2012). TLR2 forms heterodimers with TLR1, TLR 6 and play important role in identification of receptor molecules by distinguishing tri- and diacylated lipopeptides (Jann et al., 2008; Skevaki et al., 2015). The specificity of ligand binding has been explained for TLRs family 1–6 among mammalian species (Seabury and Womack, 2008). The variation in TLR2 gene enhances the risk of severe infections in humans, mice, and domestic cattle. The TLR polymorphism has been reported in livestock for disease resistance (White et al., 2003; Shinkai et al., 2006). TLRs show strong evolutionary variations that can be represented by non-synonymous (dN) and synonymous substitution rate ratios between sequences in phylogenetics (Jann et al., 2008). Single nucleotide polymorphic studies have been done for bovine TLRs 1, 3, 4, 5, 7, 8, 9, and 10 (White et al., 2003; Cargill and Womack, 2007). But few reports are available on distribution of genetic polymorphisms in TLRs 2 and 6 among bovines (Seabury and Womack, 2008). Describing genetic variation in the form of SNPs associated with disease resistance in livestock may be used as markers for genetic selection. Pahari cattle, also known as Hill or Desi cattle of Himachal Pradesh in Northern India are one of

* Corresponding author at: Department of Veterinary Microbiology, Dr. G.C. Negi-College of Veterinary and Animal Sciences, CSK -Himachal Pradesh Agricultural University, Palampur 176062, India.
E-mail address: sverma8@gmail.com (V. Subhash).

https://doi.org/10.1016/j.rvsc.2017.12.003
Received 7 March 2017; Received in revised form 20 November 2017; Accepted 14 December 2017

the non-descript indigenous breeds of India. These cattle mostly reared in outer, lower ranges of the Himalayas, about 600 to 2100 m above sea level. Large number of cattle of this non-descript breed have not yet been cross-bred with *Bos Taurus* or other recognized *Bos indicus* breeds of India. These cattle are believed to possess gene combinations and special adaptation traits such as disease resistance, adaptation to rough mountainous topography, harsh climate and ability to thrive on poor quality forages (Verma et al., 2014; Hansen, 2004; Mwai et al., 2015). In spite of such unique characteristics, these cattle are not yet characterized. These cattle have definite utility in the native tract of their distribution, but over last few decades there has been marked decline in their population due to widespread use of crossbreeding with exotic breeds. The ever-increasing number of exotic cattle germplasm has led to increase in incidence of existing diseases and emergence of new ones. It is well documented that genetic variability in genes modulating immune responses differentially affects the disease resistance and susceptibility (Porto-Neto et al., 2013). In order to enable policy makers to make informed decisions regarding conservation and improvement of this unique cattle germplasm of this region, important components that may contribute to its high disease resistance potential are required to be scientifically investigated. It is with this background; the study was conducted to sequence and compare TLR2 of selected Pahari cattle (with no past history of cross-breeding) with other indigenous and exotic breeds to determine TLR2 variation and corresponding SNPs that may have potential role in disease resistance or susceptibility.

2. Materials and methods

2.1. Sample collection

Blood samples were collected from the Pahari cattle of different geographical locations of Himachal Pradesh.

2.2. TLR2 gene amplification and sequencing

Genomic DNA was isolated from blood samples using conventional methods (Sambrook et al., 1989). Different pairs of PCR primers were designed for amplification of the complete CDS of TLR2 (Table 1). The TLR2 coding sequence was amplified in 50 μL PCR reaction mixture composed of 50 ng/μL of genomic DNA, 0.5 μM of each primer, 0.2 mM each dNTP, 1 × PCR buffer and 5 unit/μL of *Taq* DNA polymerase (Qiagen, Valencia, CA). The reaction mixtures were incubated in a thermal cycler for 10 min at 95 °C followed by 5 cycles of 30 s at 95 °C, 20 s at 60 °C, and 15 s at 72 °C and an additional 30 cycles of 20 s at 95 °C, 20 s at 58 °C, and 30 s at 72 °C, with a final extension of 10 min at 72 °C. The amplicons were separated on 1% (w/v) agarose gel and purified using Qiaquick Gel Extraction Kit (Qiagen, Germany).

The nucleotide sequencing of the purified PCR products was done using Big-Dye Terminator Cycle Sequencing Kit (Applied Biosystems, CA, USA). The PCR reaction comprised of 2–3 μL PCR product (100 ng), 1 μL primer (10 μM), 2 μL of 5X sequencing buffer and 1 μL of Big Dye™ ready reaction mix. The final volume was adjusted to 10 μL with nuclease-free water. The reaction conditions performed in thermal cycler consisted of initial denaturation at 94 °C for 4 min, followed by 25 cycles of 94 °C for 10 s, 52 °C for 40 s and 60 °C for 4 min. The PCR products were purified using post sequencing clean up method. To 10 μL of reaction mix was added 90 μL of deionized water. About 1/10 volume (100 μL) of sodium acetate (3 M) and 2.5 volumes (250 μL) of 96% ethanol were added to the reaction mix. The contents were mixed by vortexing. The tubes were placed over ice for 10 min. The contents were centrifuged and pellets were washed with 70% ethanol, air-dried and resuspended in 25 μL Hidiformamide and vortexed for 15 min. The purified PCR products were transferred to 96 well injection plates and denatured at 94 °C for 5 min. The sequencing was performed using an automated sequencer ABI 3100 DNA capillary sequencer (Applied Biosystems, Foster, CA).

Table 1
Primer pairs used for the amplification of bovine TLR2.

Primers	Sequence	Product size (bp)
TLR2_1	F: TCCTGCTCCATATTCCTACG (816 bp) R: TGACTGTGTTTGACATCATGG	816
TLR2_2	F: CTCATTCATTTATGGCTGGC (668 bp) R: GACCTGAACCAGGAGGATG	668
TLR2_3	F: AGATCACCTATGTCGGCAAC (681 bp) R: CATGGGTACAGTCATCAAACTC	681
TLR2_4	F: AGCATCCATCAGTGAAATGAG (774 bp) R: GGTAAGAAGGAGGCATCTGG	774
TLR2_5	F: AGTTTAACCCAGTGCCTTCC (730 bp) R: TGGAGTCAATGATGTTGTCG	730
TLR2_6	F: CCTACTGGGTGGAGAACCTC (436 bp) R: ACCACCAGACCAAGACTGAC	436

2.3. Sequence analysis and SNP genotyping

The sequence identification and analysis was done using BLASTN of National Center for Biotechnology Information (Altschul et al., 1990). Multiple sequence alignment was done using BioEdit v.7.1.3 (Hall, 1999). All the nucleotide sequences of the TLR2 gene among 14 bovines were compared with the available sequences in NCBI database. Both sequence strands were compared to obtain a complete sequence for identifying polymorphisms. The SNPs were detected using PolyPhred and confirmed by manual inspection. The ratio of synonymous and non-synonymous nucleotide substitutions was calculated and positions containing gaps were eliminated using the Nei-Gojobori method (Jukes-Cantor) in MEGA 6 (Tamura et al., 2013). The domain architecture in TLR2 was predicted using Simple Modular Architecture Research Tool (SMART) (Letunic et al., 2014).

3. Results

3.1. TLR2 gene sequencing and SNP genotyping

A complete gene of ~3613 bp was obtained by using primer pairs that amplified reciprocally overlapping fragments. The gene fragment composed of two exons with 136 bp 5'-UTR, 2355 bp CDS and 1122 bp long 3'UTR. The analysis of the entire CDS identified 19 new SNPs at different positions (Table 3). Among these SNPs, 47% (n = 9) were non-synonymous SNPs, 53% (n = 10) were synonymous SNPs. Of the 19 SNPs 63% (n = 12) were AG or TC, 21% (n = 4) were CA or GT, 5% (n = 1) were GC, and 10% (n = 2) were AT base changes. Seven non-synonymous SNPs were distributed in the EC domains of bovine TLR2 with one SNP in TM and TIR domain. Similarly, 4 synonymous SNPs were distributed in ECD, 2 in TM domain and 4 in TIR domain (Table 2). Five mutations N62N, W119L, F544F, R563H and H569H were located within LRRs of ECD. The observed ratio of synonymous to non-synonymous substitutions was < 1 and indicating purifying

Table 2
Percent homology of amino acid sequence of bovine TLR2 with mammalian species.

Species	GenBank accession number	Amino acid homology (%)	Identification
Bos taurus	ACH92789	99.5 (780/784)	Toll-like receptor 2
Bos indicus	ALZ41705	98.9 (776/784)	Toll-like receptor 2
Bubalus bubalis	ADO51628	98.0 (769/784)	Toll-like receptor 2
Ovis aries	CAQ37821	93.8 (736/784)	Toll-like receptor 2
Capra hircus	ADF65598	93.7 (735/784)	Toll-like receptor 2
Sus scrofa	NP998926	80.7 (633/784)	Toll-like receptor 2 precursor
Homo sapiens	NP003255	77.6 (609/784)	Toll-like receptor 2 precursor