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Comprehensive Sequence Analysis of *TLR* Genes in Vechur Cattle

R. Lakshmi[1*] and K. K. Jayavardhanan[2]

[1]Research Fellow, Department of Veterinary Parasitology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai – 600 007

[2] Professor and Head, Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, Thrissur, Kerala – 680 651

*Corresponding Author E-mail: lakshmivetbio@gmail.com

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ABSTRACT

Vechur cow, an indigenous cattle variety of Kerala, is well adapted to the hot, humid tropical climate and high disease resistance. Breed characterization is a primary step in designing appropriate management and conservation programs of indigenous cattle. This research was carried out to characterize the Toll-like receptors (TLRs) of indigenous Vechur cattle breed. DNA was extracted from blood samples and total RNA from milk somatic cells was isolated using TRI reagent from Vechur cattle. Primers used to amplify the promoter region and mRNA of TLR2, TLR4 and TLR9 genes in Vechur cattle were designed from Bos taurus sequences. The amplified promoter and mRNA product were sequenced commercially by primer walking technique. The promoter region of TLR2 of Vechur cattle with the Bos taurus sequence showed 98 per cent similarity whereas TLR4 and TLR9 revealed 99 per cent similarity. TLR2 and TLR9 revealed variations for three sequence motifs. All three TLR mRNA sequences showed 99 per cent homology with Bos taurus sequence and exposed variations for 17 nucleotide in TLR2, 7 nucleotide in TLR4 and 5 nucleotide in TLR9 mRNA. The ectodomain of Vechur cattle displayed 10 LRRs for TLR2, 13 LRRs for TLR4 and 18 LRRs for TLR9. The primary structure of protein showed highest per cent of leucine amino acid for all three TLRs and alpha helix is the prominent secondary structure seen in all TLRs followed by beta turn and random coil. The tertiary structure was dominated by LRRs to form a solenoid protein domain. These data provide the most comprehensive view of TLRs in the Vechur cattle, and provide new opportunities to better understand the regulation of TLR genes in Vechur cattle.

Key words: *Vechur, immune, TLRs, Promoters and mRNA*

INTRODUCTION

Vechur cow, an indigenous cattle variety of Kerala, a rare breed of *Bos indicus*, is the smallest cattle breed in the world. This dwarf cattle breed is well adapted to the hot, humid tropical climate of Kerala and is highly disease resistance[1]. Selection of breeds that are resistant to diseases and the integration of resistant trait in cattle is a promising alternative to decrease the problems caused by infectious diseases.

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Breed characterization is a primary step in selection of indigenous cattle for breeding programs. This study was carried out to characterize the Toll-like receptors (*TLR*s) of indigenous Vechur cattle breed. Recognition of pathogen is an essential for the initiation of innate immune system and is mediated by *TLR*s receptors[2]. *TLR*s are critical sensors of invading pathogens and effectors of the innate immune system mechanism, which enables the host to eliminate pathogens. Currently, 13 *TLR*s have been identified in mammals of which ten are known to occur in cattle, each possessing their own ligand recognition site and the expression of *TLR* transcripts varies among different mammalian species[3]. Among the members of *TLR* family, the *TLR*2 is the key receptors for PAMPs from Gram-positive bacteria and *TLR*4 for Gram-negative bacteria. *TLR*9 acts as an important adjuvant and complement the selection of *TLR*s for the mounting of the immune response[4]. To investigate the role of innate immune system of Vechur cattle in resisting diseases, members of mRNA of *TLR* and its promoter regions were sequenced and characterized.

MATERIALS AND METHODS

Sample

Blood and milk samples of Vechur cattle breed were collected from the Vechur Conservation unit, Mannuthy Thrissur. Blood samples were collected from jugular vein of Vechur cattle, using sterile needles and syringes with 1ml of acid citrate dextrose anticoagulant for 10 ml of blood.

DNA Isolation

Genomic DNA was isolated from blood sample by using phenol chloroform method [5]. The concentration, purity and quality of DNA were checked in a nanodrop spectrophotometer (NanoDrop^{TM} 2000C). The quality of the extracted genomic DNA was assessed by agarose gel (one per cent W/V) electrophoresis and by visualizing under UV trans-illuminator.

RNA Isolation

Somatic cells were isolated from the collected milk samples. Total RNA from milk somatic cells was isolated using TRI reagent The RNA isolated was treated with DNase enzyme (Sigma-Aldrich) to remove DNA contamination in total RNA. Quality and integrity of isolated total RNA was checked electrophoretically by agarose gel (1 per cent W/V). First strand of cDNA was synthesized using RevertAid First strand cDNA synthesis kit \using oligo dT primers.

Primers and PCR amplification

Primers used to amplify the promoter region of *TLR*2, *TLR*4 and *TLR*9 genes in Vechur cattle were designed using online software Primer 3 software from *Bos taurus* sequences (AC_000174, AC_000165 and AC_000179 receptively) available in National Centre for Biotechnology Information (NCBI) database. Similarly, primers for *TLR*4 and *TLR*9 genes were designed in two and three sets, respectively utilizing *TLR*4 (Accession No. NM_174198.6) and *TLR*9 (NM_183081.1) genes sequence of *Bos taurus*. As *TLR*2 gene of Vechur has been was sequenced earlier and it was retrieved directly from the NCBI Genbank (Accession No. KT862891.1). Primer properties were analysed using sequence manipulation suite (http://www.bioinformatics.org/sms2/pcr-primer_stats.html). The conditions for PCR were standardized to minimize non-specific amplification and to get maximum amplification of the desired product. Annealing temperature was standardized for the promoter region for *TLR*2, *TLR*4 and *TLR*9 as follows 53.2°C for 30 sec, 56.0°C for 30 sec, 53.0°C for 30 sec, respectively. The amplification reaction was carried out in 0.2 ml PCR tubes with 40 µl of reaction volume.

Sequence Analysis

The amplified product of *TLR* prompters were sequenced by Sanger's enzymatic method. The mRNA of *TLR* was sequenced by primer walking technique (Chromous Biotech, Pvt. Ltd, Bangalore). Complete sequence of each gene was obtained by multiple sequence alignment (MSA) technique using ClustalW method in MegAlign DNASTAR. Nucleotide sequences of promoter region of *TLR* genes were submitted to NCBI GenBank are

assigned with the following accession numbers: KR559022.1 (*TLR*2), KR559023.1 (*TLR*4) and KR559024.1 (*TLR*9). Similarly, the mRNA sequence of *TLR* genes were assigned with the following accession numbers: KX138607 (*TLR*4) and KX138608 (*TLR*9). The sequences of *TLR*2, *TLR*4 and *TLR*9 genes of Vechur cattle were analysed using various bioinformatics tools.

RESULTS AND DISCUSSION

Vechur cattle breed of Kerala are well known for their low susceptibility to diseases than the crossbred cattle. The marked differences in susceptibility to diseases predict that there is considerable variation in the efficiency of the antimicrobial defence within the Vechur breeds. However, the mechanisms underlying attenuated or exaggerated response of Vechur breed to disease resistance is remain unclear. As *TLRs* are critical for innate immunity, exploring the genetic basis of varied expression of *TLR* and function may unravel many aspects of *TLR* signalling. *TLR*s are critical sensors of invading pathogens and effectors of the innate immune system mechanism, which enables the host to eliminate pathogens. *TLR*2, *TLR*4 and *TLR*9 are reported as critical sensors of innate defence against bacterial infection[6]. Hence, we sequence the promoter and mRNA of *TLR*s to elucidate innate immune system of Vechur cattle, and therefore characteristics Vechur *TLR* sequences were analysed comprehensively and reported here.

Promoter sequence of *TLRs*

The promoter region of *TLRs* plays a crucial role in the transcription of genes. Promoter region consists of various consensus sequences, capable of regulating the rate of transcription by inducing or suppressing the respective genes. The promoter sequence obtained for *TLR*s genes in Vechur cattle were assessed with that of *Bos taurus* for the important sequence motifs, consensus sequence and their functions (Table 1). The sequence comparison of *TLR*2 revealed 98 per cent similarity with *Bos taurus* sequence and showed variations at three sequence motifs in TATA, Cyclic responsive element (CRE) and Sp1 binding site. The promoter sequence of *TLR*4 of Vechur cattle shown 99 per cent similarity with that of *Bos taurus* sequence without any significant variations in motif regions. Promoter region of *TLR*9 also showed 99 per cent similarity to *Bos taurus* genome and revealed changes at three sequence motifs in CAAT, NF-κB, Sp1 binding site regions required for regulation of transcription. A compressive characterization of promoter sequence of TLRs genes in Vechur cattle has been reported earlier[7]. The reported variation for promoter sequence of *TLR* genes in Vechur breed might influence the innate immunity response against disease resistance.

mRNA sequence of *TLRs*

Nucleotide sequence of *TLR*2 mRNA in Vechur cattle was obtained from the Genbank revealed ORF of 2355 bp coding of 784 amino acids. Sequence of *TLR2* revealed 2355 bp ORF in Vechur cattle, which was in accordance with Raja *et al*[8]. Nucleotide sequence of *TLR*4 mRNA in Vechur cattle revealed ORF of 2526 bp coding of 841 amino acids and *TLR*9 revealed ORF of 3090bp coding for 1029 amino acids. All three *TLR* mRNA sequences showed 99 per cent homology with *Bos taurus* sequence and exposed variations for 17 nucleotide in *TLR*2, 7 nucleotide in *TLR*4 and 5 nucleotide in *TLR*9 mRNA (Table 2).

Amino acid sequence of *TLR*s

Amino acid sequence of Vechur *TLR*s protein was predicted and analyzed by using SMART. The ectodomain of Vechur cattle displayed 10 LRRs for *TLR*2, 13 LRRs for *TLR*4 and 18 LRRs for *TLR*9. Raja *et al*.[8] reported 22 LRR for *TLR*4 gene in goat and he also observed variation in the predicted numbers of LRR. The LRR domain is important for recognition of specific pathogen, since change in the structural variation in LRR domain might be one of the reason for better immunity in Vechur cattle. Prediction of signal peptide sequence is important for translocation of the newly synthesized protein and it is further cleaved by signal peptidase enzymes. Signal

peptide prediction exposed the presence of signal peptide with high prediction score of 0.8 out of one for *TLR*2, 0.6 for *TLR*4 and 0.7 for *TLR*9. Cleavage site between position 20 and 21 for *TLR*2**,** 16 and 17 for *TLR*4, 24 and 25 amino acid for *TLR*9 (Table 3). In *TLR*2, the signal peptide cleavage site was predicted between 20th and 21st amino acid, similar to the report of Banerjee *et al*[9].

The primary structure of protein showed highest per cent of leucine amino acid for all three *TLR*s, 15.43 for *TLR*2, 16.05 for *TLR*4 and 18.56 for *TLR*9. Leucine rich regions recognize various microbial components engaging signaling cascade that results in the response against such microbes. These results are in accordance with the Raja *et al*[7]. Alpha helix is the prominent secondary structure seen in all *TLR*s followed by beta turn and random coil. Highest per cent of alpha helix for *TLR4* gene in goat was reported by Goyal[10]. Tertiary structure for *TLR*s protein was predicted using SWISS MODEL. The tertiary structure of *TLR* protein had high level of quality score with P-values less than 0.002. The structure was dominated by leucine rich repeats to form a solenoid protein domain. The functional domains have been distinguished by different colors (Figure 1). The assembled domain composed of many such repeats, each repeat unit has beta strand-turn-alpha helix structure.

Table 1: Variations observed in sequence motifs of *TLR* promoters

Motif	Consensus Sequence	*TLR*2		*TLR*9	
		Bos taurus	**Vechur**	***Bos Taurus***	**Vechur**
TATA	TATAAA	TATAA	T**C**TAA	No variation	No variation
CRE	TGACGTCA	TGA**AT**TCA	TG**GAT**TCA	-	-
CAAT	CAAT	No variation	No variation	CA**G**T	CAAT
NF-κB	GGGRNNYYCC, R-purine, Y-pyrimidine.	No variation	No variation	GGCA**G**TCATC	GGCA**A**TCATC
Sp-1site	GGGCGG	**A**GGCG	**G**GGCG	GGGGC**A**G	GGGGCGG
Note: *TLR*4 doesn't revealed any variation for motif region					

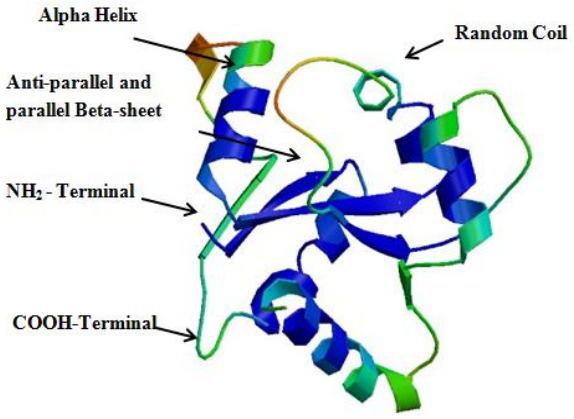
Table 2: Nucleotide sequence variation of *TLRs* gene in Vechur cattle

Gene	Nucleotide Variation	Synonyms Substitution	Non-synonyms Substitution
***TLR*2**	17	9	8
***TLR*4**	7	3	4
***TLR*9**	5	3	2

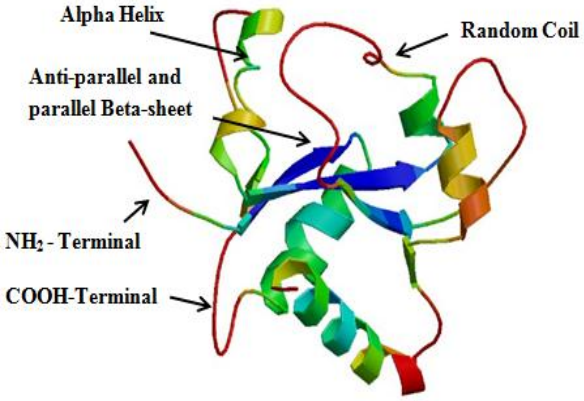
Table 3: Signal peptide region and LRR of *TLR*s

Gene	Signal Peptide	LRR
***TLR*2**	Between 20 and 21 amino acid	10
***TLR*4**	Between 16 and 17 amino acid	13
***TLR*9**	Between 24 and 25 amino acid	18

Tertiary structure of *TLR2*



Tertiary structure of *TLR4*



Tertiary structure of *TLR9*

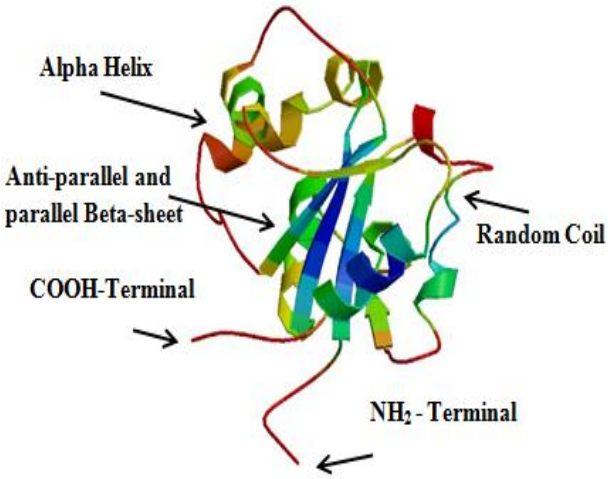


Fig. 1: Tertiary structure of *TLR*s Protein in Vechur Cattle

CONCLUSION

These data provide the most comprehensive view of *TLR*s in the Vechur cattle. The presence of unique structural features and substantial variation for *TLR* genes in Vechur cattle breed, may change the confirmation of *TLR* proteins, which may influence the binding affinity and interaction with pathogen to boost the innate host disease resistance in Vechur cattle breed. Our data, in combination with other data, provide new opportunities to better understand the regulation *of TLR* genes in Vechur cattle.

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