SEQUENCE ANALYSIS OF TOLL-LIKE RECEPTOR9 GENE IN VECHUR CATTLE

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ABSTRACT

Toll-like receptors (TLRs) are considered as key sensors to trigger the host innate and adaptive immune responses by recognizing various PAMPs and initiating signal transduction. *TLR9* mediates the recognition of unmethylatedCpG dinucleotide motifs commonly found in both bacterial and viral genomes.Vechur cattle, a rare breed of *Bosindicus*, are highly disease resistant compared to crossbreed animals.The study was undertaken to characterize the *TLR9* gene in Vechur cattle to provide an insight into the mechanisms involved in disease resistance. Nucleotide sequence of *TLR9* mRNA in Vechur revealed ORF of 3090 bp coding of 1029 aminoacids. Sequence analysis of *TLR9*mRNA of Vechurwith *Bostaurus*sequence showed 99 per cent homology and exposed 5 nucleotide variations (2 non-synonymous and 3 synonymous substitutions). EighteenLRR domains were identified for *TLR9* in Vechur cattle, of which LRR17 domain showed variation for one amino acid. Protein composition showed highest per cent of leucine (18.56 %) and serine (8.84%) amino acid and structure displayed contributing of alpha helix (40.72%), beta turn (19.05 %) and random coil (40.23 %). Phylogenetic tree showed all bovidae family falling under the same group, indicated conserved nature of *TLR9* gene. Sequence variations observed for *TLR9* gene in the Vechurcattle make thisgene alikely candidatefor studyingvariations observedinthe resistance to disease at the phenotypic level.

Key words: Vechur breed, disease resistant, *TLR9*and sequence

INTRODUCTION

Toll-like receptors are highly conserved cell receptors that form a critical component of thehost's innate immune system and adaptive immune responses by recognizing various PAMPs and initiating signal transduction (Rankin *et al.,* 2001; Roach *et al*., 2005). These receptors are vital in the containment, and eventual clearance of a number of diseases (Rutz*et al*., 2004; Bhan*et al*., 2008), and are under scrutiny for their impact on the induction of Th1 *vs.* Th2 responses of the specific immune system (Kline *et al*., 1998; Dorn and Kippenberger, 2008). Different TLRs recognize the unique molecular signatures of microbes and trigger the innate immune system. *TLR9* is a pattern-recognition receptor present inside the cells, involved

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in immune signalling and plays an essential role through recognition of various componentsof bacteria. *TLR9* has been demonstrated as a receptor for bacterial DNA containing unmethylatedCpGdinucleotides and specific sequence pattern (Hemmi*et al*., 2000). Specifically, *TLR9*implicated as a link among the innate and adaptive immune system by favouring immune response and enhancing auto-antibody production.*TLR9* is predominantly localized in lysosomes, where the low pH promotes specific *TLR9* binding to unmethylated DNA (Macfarlane and Manzel, 1998; Latz *et al.,*. 2004; Rutz *et al.,* 2004). Bovine *TLR9* is located on BTA22. The *TLR9* mRNA consists of two exons and is 3255 bp including 5′ and 3′ UTRs, The genomic size of *TLR9* is 4264 bp and the protein is 1029 aa (Cargill and Womack, 2007).

Vechur cattle, a rare breed of *Bosindicus*, is an indigenous breed of Kerala and it is the smallest cattle breed in the world. They are well adapted for the hot, humid tropical climatic conditions of Kerala and are highly disease resistant compared to other crossbred animals.Since TLRs appear to be crucial in the natural immune response, therefore we report here the sequence analysis of *TLR9*gene in Vechurcattle that might be helpful toward a better understanding of innate immunity of Vechur breed.

MATERIALS AND METHODS

RNA isolation and cDNA synthesis

Milk samples were collected from three cattle of Vechur breed maintained in the conservation unit at College of Veterinary and Animal Sciences, Thrissur, Kerala. Somatic cells were isolated from the collected milk samples.The average somatic cells, in collected milk samples are 0.54 x 10^5 cells/ml. Total RNA from milk somatic cells was isolated using TRI reagent and digestion with DNase enzyme (Sigma-Aldrich) to remove any DNA contamination. Quality and integrity of isolated total RNA was checked electrophoretically by agarose gel (1 per cent W/V) prepared in 1X TBE buffer and concentrations were determined by spectrophotometerically (NanoDrop™ 2000C). First strand of cDNA was synthesized from the isolated RNA using RevertAid First strand cDNA synthesis kit.

Primer designing, synthesis and dilution

Primers used for amplification of *TLR9*gene was designedin three sets, using Primer 3 software utilizing*TLR9* (NM_183081.1) gene sequence of *Bostaurus*. Primer properties were analysed using sequence manipulation suite. The three sets of designed forward and reverse primers were custom synthesized at Sigma Aldrich, India (Table1).The primers were reconstituted in nuclease free water to a concentration of10 pM/µl.

Standardization of PCR conditions

The PCR reaction mixtures contained 9µlof pure sterile water, 25µl of 2X PCR master mixes, 2µl each of forward and reverse primers and 2µl of Template cDNA. The conditions for PCR were optimized for annealing temperature using gradient PCR technique and the annealing temperature showing optimum amplification without non-specific product was selected (Table 2).

Sequencing of amplified product

The amplified products(forward and reverse)were sequenced commercially (Chromous Biotech, Pvt. Ltd, Bangalore) by primer walking technique and final complete sequences were obtained in FASTA format along with respective ABI files containing chromatogram. Complete sequence of the gene was obtained by multiple sequence alignment (MSA) technique using ClustalW method in MegAlign DNASTAR.

Sequences analysis

Sequence obtained for *TLR9* gene in Vechur cattlewas analysed using various bioinformatics tools. The Basic Local Alignment Search Tool (BLAST) was used to compare the nucleotide or protein sequences with sequence databases directly. Simple Modular Architecture Research Tool (SMART) was employed in the prediction of Leucine rich repeats (LRR). SignalP 4.1 server was used to predict the presence and location of signal peptide cleavage sites in amino acid sequences (Petersen *et al*., 2011). Phylogenetic trees wereconstructed usingDNAstarLasergeneMegAlign program. DNASTAR Protean was used for prediction of protein primary structure. PSI PRED and SOPMA were used to predict the number of alpha helices, beta turns, random coils and extended strands in the predicted *TLR9*protein.

RESULTS AND DISCUSSION

Vechur cattle, an indigenous breed of Kerala is highly disease resistant compared to crossbreeds. Since *TLR9* appear to be crucial in the natural immune response, this study was to characterize the sequence of *TLR9*gene in Vechurcattle, that would be helpful for better understanding of the innate immune mechanism in Vechur breed.

TLR9 gene in Vechur cattle was amplified with three set ofdesigned primers. The sizes of amplified products were verified using agarose gel electrophoresis (Figure 1).The three set of amplified product of *TLR9*gene weresequenced by primer walking technique and sequenceswere obtained in FASTA format. Complete sequence of *TLR9*gene was assembled by combining the sequence of set I, II and III by multiple sequence alignment.The full length *TLR9*cDNAsequence of Vechur has been submitted to NCBI Genbank(Accession No. KX 138608).

Nucleotide and protein sequence

The assembled sequence (3158bp) revealed 5' UTR from 1-53, CDS from 54 to 3143 and 3'UTR from 3144 to 3158 positions. Two nucleotidescausingnon-synonymous substitution and three nucleotides causing synonymous substitution in coding region were identified. The variations were observed at the positions *viz*., G1892C, G2168C, C2468T, T2846C and C2854A. Banerjee *et al*. (2012) also reported one nucleotide transitions in*TLR9*coding region of buffalo.

Nucleotide sequence contained an ORF of 3090bp coding for 1029 amino acids. Same number of amino acids for *TLR9* gene was also reported in Holstein cows by Mahmoudzadeh*et al*.(2014). Vechurcattle *TLR9*protein showed 99 per cent homology with *Bostaurus*. The predicted amino acid sequence revealed non-synonymous variation at 705th position where glutamic acid was substituted with aspartic acid, and at 934th position where alanine was substituted with glutamic acid.

Physico-chemical properties and protein structure

Amino acid sequence of Vechur *TLR9* protein was analyzed by using SMART (Figure 2)which revealed amino acids of ectodomain (1-774), cytoplasmic domain (775-1029)and TIR domain (865-1012). The ectodomain of Vechur*TLR9*displayed different regions, which included 18leucine-rich repeats (LRRs), and a conserved toll/ interleukin-1 receptor domain (TIR). There was change in single amino acid at LRR17 domain of Vechur cattle when compared to *Bostaurus*, which might enhance immune response in Vechur cattle. Specific molecular recognitionis regularly achieved by interactions facilitatedthrough the variable residues of side chains,contributed by each LRR(Gay and Gangloff, 2007).

Hydrophobic amino acid, leucine was found to be major amino acid (18.56 per cent) in the primary structure of *TLR9*protein (Table 3). The second major amino acid was found to be serine (8.84 percent). These two amino acids are involved in post-translational modification and functional activities. The hydrophilic amino acid residues will form hydrogen bonds with each other and also with other water soluble molecules (Pragathi, 2015). The result of our study indicates the presence of 276 polar amino acids which may participate within protein and between protein interactions. *TLR9* gene has the isoelectric point of 8.80 in Vechur cattle, which could be due to more number of basic amino acids (102) compared to acidic amino acids (82).

Signal peptide prediction exposed the presence of signal peptide with high prediction score of 0.7 out of one. Cleavage site between position 24 and 25amino acid sequence revealed the presence of 24 amino acid residues of the signal peptide and 1005 amino acid residues of the mature polypeptide chain (Figure 3). Banerjee*et al.* (2012) also reported cleavage at 24th position of *TLR9*peptide comprising 24 amino acid residues as signal peptide sequences in buffalo.

Primary structure of the protein with respect to composition of amino acid revealed that highest number of hydrophobic amino acids consisted of 191 leucine residues in peptide sequence (Table 4) which may contribute to helix formation in the secondary structure of protein and moreover, it is involved in LRR in ectodomain of *TLR9*. Secondary structure was predicted by using PSI PRED (Figure 4)displayed portions of amino acid sequence contributing to formation of alpha helix (40.72 per cent), beta turn (19.05 per cent) and randomcoil (40.23 per cent).

Sequence homology and phylogenetic relationship

BLASTn was used to retrieve the *TLR9* nucleotide sequences of mammalian species and similarity were analysed with Vechur cattle.*TLR9*ofVechurcattle showed maximum identity with *Bostaurus and Bosgrunniens* sequence (99.8 per cent) and least identitywith *Susscrofa*(87.4 per cent).

The similarityanalysis for predicted amino acid sequences of *TLR9*revealed that peptide sequences of Vechur cattle has maximum per cent identitywith *Bosgrunniens and Bison bison*peptide sequence (99.9 per cent) and least identity(79.8 per cent) with *Homo sapiens*. The phylogenetic tree analysis of nucleotide and protein sequence of *TLR9* showed that Vechur is closely related to *Bovidae* family which indicate the slow basal rate of evolution among species, while the others formed a separate branch indicating the strongly conserved nature of *TLR9*(Figures 5 and 6).

CONCLUSION

The presence of substantial variation and unique structural features for *TLR9* gene in Vechurcattle indicates the native breed specificity. Variations observed for *TLR9* gene in the Vechur cattle make this gene as a likely candidate for differences observed at the morphological level attributed to variations in the innate resistance to various bacterial infections. The polymorphism data generated has the potential for association analysis with disease resistance and susceptibility.

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Table 1: Primers designed for amplification of TLR9gene in Vechur cattle

Primer information		Sequences (5’→3’)	Product size
SET I	Forward	AAGCCGCATTCCCTGTCAT	1913 bp
	Reverse	GCAGCTTGGGGAAGTTCTTT	
SET II	Forward	CAGGGAGTGCCCAAAGAACT	757 bp
	Reverse	CACGAACTGGGAGCCATTA	
SET III	Forward	ATCTCACAGGCGGTTAATGG	
	Reverse	GGCAGGCAGTCTGTGCTATT	1590 bp

Table 2:Optimized PCR conditions for amplification of *TLR9*gene

TLR9 gene	Initial denatu-ration	35 cycles of						Final extension
		Denaturation		Annealing		Extension		
		Temp	Time	ºC	Time	Temp	Time	
SET I	95ºC for 3 mins	95	30 sec	63.2	20 sec	72	2 min	72ºC for 6 min
SET II			30 sec	63.6	30 sec		2 min	
SET III			35 sec	61.6	25 sec		2 min	

Table 3:Amino acid composition of predicted *TLR9* protein

Sl No.	Amino acids	Number	Per cent	
			Weight	Frequency
1	Ala	69	4.25	6.71
2	Cys	25	2.23	2.43
3	Asp	48	4.78	4.66
4	Glu	34	3.80	3.30
5	Phe	51	6.50	4.96
6	Gly	55	2.72	5.34
7	His	37	4.39	3.60
8	Ile	24	2.35	2.33
9	Lys	26	2.89	2.53
10	Leu	191	18.72	18.56
11	Met	13	1.48	1.26
12	Asn	56	5.53	5.44
13	Pro	57	4.79	5.54
14	Gln	38	4.22	3.69
15	Arg	76	10.28	7.39
16	Ser	91	6.86	8.84
17	Thr	44	3.85	4.28
18	Val	58	4.98	5.64
19	Trp	14	2.26	1.36
20	Tyr	22	3.11	2.19

Table 4: Nature of amino acid of predicted*TLR9*protein

Amino acid(s)	Number	Per cent	
		Weight	Frequency
Charged (RKHYCDE)	268	31.48	26.04
Acidic (DE)	82	8.58	7.97
Basic (KR)	102	13.16	9.91
Polar (NCQSTY)	276	25.80	26.82
Hydrophobic (AJLFVW)	407	39.05	39.55

Figure 1: Agarose gel image displaying PCR product size of *TLR9*gene in Vechur cattle

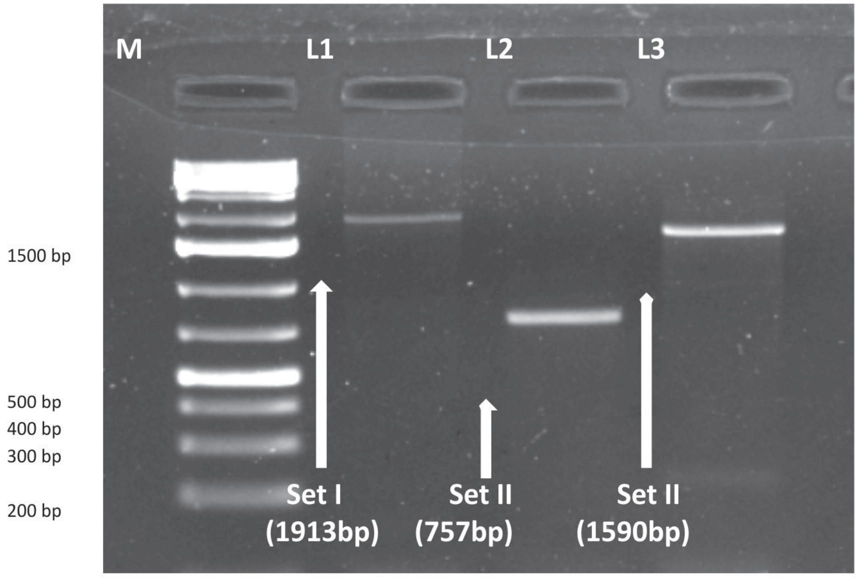

Figure 2: Predicted domain structure of Vechur *TLR9* by SMART analysis

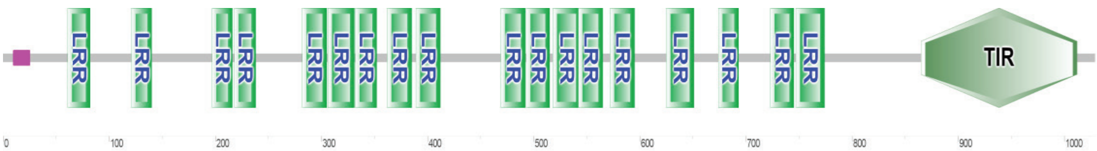

Figure 3: Signal peptide prediction for *TLR9* protein sequence

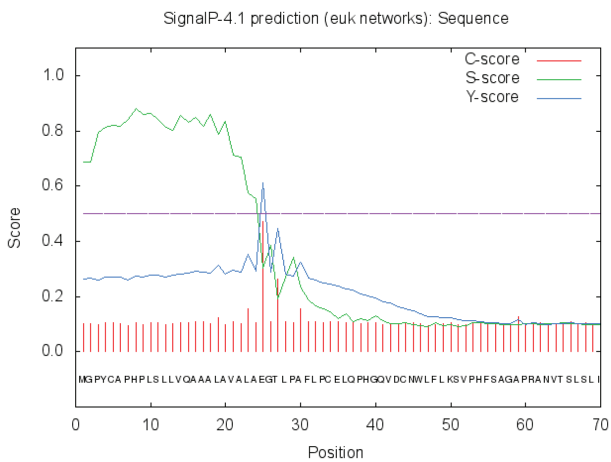

Figure 4:Predicted secondary structure of *TLR9* protein

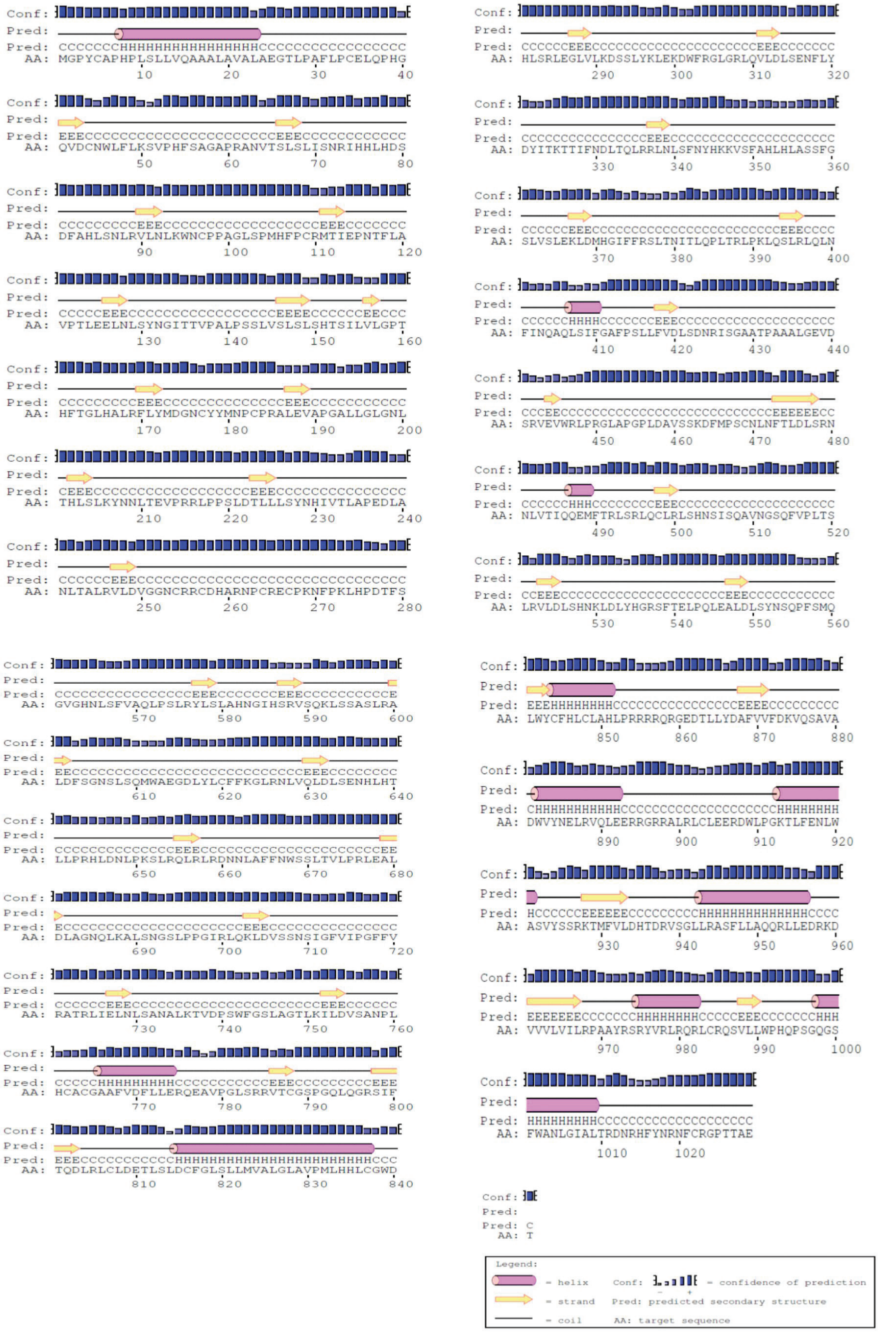

Figure 5:Phylogenetic tree fornucleotide sequence of *TLR9 gene*

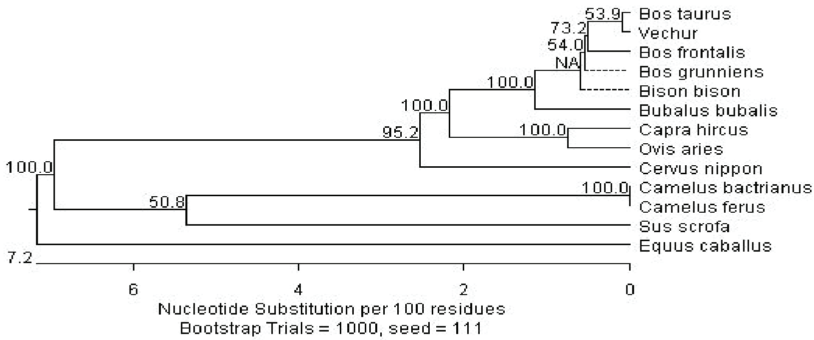

Figure 6:Phylogenetic tree for protein sequence of *TLR9 gene*