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Sequence Characterization and Phylogenetic analysis of *TLR4* Gene in Vechur Cattle

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ABSTRACT

Vechur cattle, an indigenous breed of Kerala are highly disease resistant. Toll-like receptors (TLRs) perform a vital role in disease resistance through their recognition of pathogen associated molecular patterns (PAMPs). In this study, the mRNA of Toll-like receptor 4 (*TLR4*) gene of Vechur cattle was sequenced, characterized and compared with other species. Total RNA from milk somatic cells of Vechur cattle was isolated and converted into cDNA using reverse transcriptase kit. The cDNA of *TLR4* gene was amplified with the designed primers and the product was sequenced by primer walking technique. The mRNA sequence of *TLR4* spans 2766 bp with an open reading frame (ORF) of 2526 bp coding for 841 amino acids. *TLR4* sequence of Vechur cattle with *Bos taurus* sequence showed 99 per cent homology and exposed 7 nucleotide variations (4 non-synonymous and 3 synonymous substitution). The ectodomain of Vechur *TLR4* displayed 13 LRRs and change in amino acid at LRR5 domain was observed when compared to *Bos taurus*. Leucine (16.05 per cent) and serine (8.08 per cent) were found to be major amino acids contributing to primary structure of *TLR4* protein. Secondary structure displayed portions of amino acid sequence contributing to formation of alpha helix (50.18 per cent), beta turn (17.60 per cent) and random coil (32.22 per cent). Tertiary structure of *TLR4* protein revealed distinctive horseshoe fold, composed of repeated strand-turn-alpha helix structure. Phylogenetic tree of *TLR4* gene showed that Vechur cattle closely related to *Bos taurus*. The observed structural variation in the mRNA sequence of *TLR4* gene in Vechur cattle breed will provide an important basis for further study on the relationship between polymorphisms and host disease resistance.

Keywords: Vechur breed, immunity, *TLR4*, sequence and phylogeny

The 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. Vechur cattle, an indigenous breed of Kerala and smallest cattle breed of *Bos indicus*, are well adapted for the hot, humid tropical climatic conditions of Kerala and are highly disease resistant. Characterization of the immune system of resistant Vechur breeds might provide an insight into the mechanisms involved in disease resistance and helpful to the breeding programmes for selection of resistant breeds.

Toll-like receptors (TLRs) are involved in the stimulation of the immune system in response to an infection and the best-described innate receptors. TLR performs a vital role in disease resistance through their recognition of pathogen associated molecular patterns (PAMPs) and provides critical host defence during microbial infection (Medzhitov, 2007). TLRs recognize the unique molecular signatures of microbes and trigger the innate immune system (Ishii *et al.,* 2008). *TLR4* was the first mammalian TLR described, and it is correspondingly the best described of the family of TLRs (Takeda *et al.,* 2003). *TLR4* is part of a group of evolutionarily conserved pattern recognition receptors involved in the activation of the immune system in response to different pathogens, including fungi, viruses and bacteria and plays an important role in the regulation of neutrophil life span (Sabroe *et al.,* 2003), and migration of polymorphonuclear leucocytes by regulating cell surface chemokine receptors (Fan *et al.,* 2003). It is also involved in the recognition of microbial antigens (Weiss *et*

al., 2007) mediating cytokine production and stimulation of host defence (Ferwerda *et al.,* 2007). *TLR4* also has been shown to play an important role in resistance to *Streptococcus pneumoniae* - induced pneumonia (Malley *et al.,* 2003). Role of *TLR4* in the innate immune response of cows during periods of risk from intramammary infection by Gram- negative organisms was also reported (Miller *et al.,* 2005).

In this study, we sequenced the mRNA of *TLR4* gene in the Vechur cattle and analyzed the characteristics of its nucleotide and amino acid sequences and its phylogenetic pattern with other species. This study will provide an important basis for the further study on the structural variation of the *TLR4* gene and its relationship to disease resistance.

MATERIALS AND METHODS

Ethical Approval

The study was approved by the research committee framed by the University. Ethical approval was not required, as no life animals were used in this study. However, adequate measures were taken to minimize pain or discomfort in accordance with the International Animal Ethics Committee.

Samples

The samples for this study were collected from Vechur cattle breed maintained in the Vechur conservation unit, Thrissur. The entire internal house environment was disinfected. The milk samples were collected in three Vechur cattle in sterile centrifuge tube. Somatic cells were isolated from collected milk samples.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from milk somatic cells using TRIzol reagent of SIGMA (As per the manufacturer's protocol). The quality of extracted RNA was determined by agarose gel electrophoresis (1.5%) and ethidium bromide staining. Residual DNA was removed from extracted total RNA by treatment with DNase1. The concentration of RNA was assessed by using nanodrop spectrophotometer. The isolated total RNA was subjected to first strand cDNA synthesis by using oligo dT primers (Thermo Scientific, K1622).

Total RNA from milk somatic cells was isolated using TRI reagent and treated with DNase enzyme (Sigma-Aldrich) to remove DNA contamination. Quality and integrity of total RNA was checked electrophoretically by agarose gel (1 per cent W/V) prepared in 1X TBE buffer and spectrophotometerically using NanoDrop™ 2000C. RNA sample was subjected to cDNA synthesis with the RevertAid™ First-Strand cDNA Synthesis Kit.

Primer designing and synthesis

Coding DNA sequences (CDS) for the *TLR4* (Accession No. NM_174198.6) of *Bos taurus* was downloaded from the NCBI GenBank (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Primers used for amplification of *TLR4 gene* in Vechur sample was designed in two sets, using Primer 3 software (http://simgene.com/Primer3). Primer properties were analysed using sequence manipulation suite. The designed primers were synthesized and procured from Sigma-Aldrich in desalted form (Table 1). Primers were reconstituted in nuclease free water to have a 100pM/µl stock solution, further 10 pM/µl of primer working solution was prepared.

Table 1: Primers designed for amplification of *TLR4* gene

Primer information			Sequences (5'→3')	Product size
TLR4	SET I	Forward	CGGCACAGACAGAGGGTTAT	1538 bp
		Reverse	TGTGAAGATGTCAGGGAGCA	
	SET II	Forward	CAGAAACCTCCGCTACCTTG	1309 bp
		Reverse	CTCGCGTACCACTGAATCAC	

Standardization of PCR

The PCR reaction was carried out in 0.2 ml PCR tubes with 40 µl of reaction volume consists of 2.0 µl cDNA template (1000 ng/µl), 25 µl PCR master mix (2X), 4.0 µl of forward and reverse primers (10 pM/µl), and 9 µl of nuclease free water. The conditions for PCR were

Table 2: PCR conditions for amplification of *TLR4* gene

TLR4 Gene	Initial Denaturation	35 cycles of						Final Extension
		Denaturation		Annealing		Extension		
		ºC	time	ºC	time	ºC	time	
Set I	95 ºC for 3 mins	95	35 sec	62.0	30 sec	72	2 min	72ºC for 6 min
Set II			30 sec	59.2	30 sec		2 min	

optimized for annealing temperature using gradient PCR technique and the annealing temperature showing optimum amplification with absence of non-specific product was selected (Table 2).

Sequence Analysis

The amplified product of *TLR4* gene in Vechur was sequenced commercially (Chromous Biotech, Pvt. Ltd, Bangalore) by primer walking technique. Final complete (forward and reverse) sequences were obtained in FASTA format was analysed using various bioinformatics tools.

CDS and UTR regions of *TLR4* gene were predicted using NCBI data base (http://www.ncbi.nlm.nih.gov/gene). The base composition and homology analysis of the *TLR4* gene coding region sequence were performed using the BioEdit Software (http://www.psc.edu/biomed/genedo). Multiple sequence alignment using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and construction of phylogenetic tree of *TLR4* genes were done with MegAlign program of the DNASTAR software. The SMART (http://smart.Embl-heidelberg.de/) was employed in the prediction of leucine rich repeats (LRR). SignalP 4.1 server (http://www.cbs.dtu.dk/services/S ignalP/) was used to predict the presence and location of signal peptide cleavage sites in amino acid sequences.

DNASTAR Protean was used for analysis of primary structure, while PSI PRED (http://bioinf.cs.ucl.ac.uk/psipred/) and SOPMA (https://npsa-prabi.ibcp.fr/t) were used for the prediction of secondary structure of *TLR4* protein. Tertiary structure of *TLR4* protein was prediction using SWISS MODEL (http://swissmodel.expasy.org/interactive). Ramachandran plot was carried out using RAMPAGE to assess stability of protein structure (Lovell *et al.,* 2002).

RESULTS AND DISCUSSION

Nucleotide Sequence of *TLR4* Gene

The expected size of amplified products of *TLR4* gene in two sets was verified using agarose gel electrophoresis (Fig. 1).

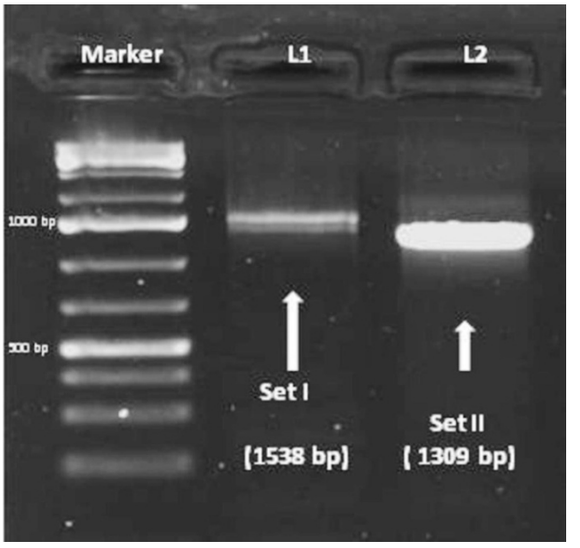

Fig. 1: Amplified product size of *TLR4* gene in Vechur cattle

Marker – 1kb plus DNA ladder; L1 – Set I of *TLR4* gene product; L2 – Set II of *TLR4* gene product

The cDNA sets of *TLR4* gene was sequenced and complete coding sequence of *TLR4* gene was obtained by multiple sequence alignment of two overlapping fragments. The assembled sequence of *TLR4* was compared with corresponding sequences from bovine and other species through BLAST analysis. CDS and UTR regions were predicted using NCBI data. The assembled sequence

(2766 bp) revealed 5' UTR from 1-120, CDS from 121 to 2646 and 3' UTR from 2647 to 2766 bp. The full length cDNA sequence of Vechur *TLR4* has been submitted to NCBI Genbank (Accession No. KX 138607).

The *TLR4* sequence of Vechur cattle with *Bos taurus* reference sequence showed 99 per cent homology and exposed seven nucleotide variations in coding region, three are synonymous and four are non-synonymous substitution. The variants are noticed at the position of T309G, A424G, T468C, T516C, A735G, A852G and T1278C (Table 3). Reported *TLR4* variants are situated in several alternative transcripts. Similarly, Banerjee *et al.* (2012) reported ten non-synonymous substitutions in *TLR4* coding region of buffalo. Highly polymorphic nature of bovine *TLR4* has been reported with more than 40 SNPs, which gives an average of one SNP per 90 bp (Panigrahi *et al.,* 2014).

Table 3: Variations observed for *TLR4* gene of Vechur cattle with *Bos taurus*

Sl. No	Nucleotide		Substitution	Amino acid	
	Position	Variation		Position	Variation
1	309	T > G	Non-synonymous	63	F > L
2	424	A > G	Synonymous		
3	468	T > C	Synonymous		
4	516	T > C	Non-synonymous	132	V > A
5	735	A > G	Non-synonymous	205	N > S
6	852	A > G	Non-synonymous	244	K > T
7	1278	T > C	Synonymous		

Protein Sequence of *TLR4* Gene

Nucleotide sequence contained an ORF of 2526 bp coded for 841 amino acids. A comparison of the deduced amino acid sequence of Vechur *TLR4* with *Bos taurus,* buffalo, goat and human is presented in Table 4. Vechur cattle showed 99 per cent homology with *Bos taurus*. The predicted amino acid sequence reveals non-synonymous variation (F63L, V132A, N205S and K244T). Primary structure of protein with respect to composition of amino acid revealed highest frequency (36.11 per cent) of hydrophobic amino acids, which found to form the transmembrane region across the lipid bilayer. The signal peptide is typically between 15 and 40 amino acids long and is essential for protein secretion, and is then subsequently cleaved from the mature protein. The signal peptide potential for *TLR4* protein sequence was analyzed using prediction algorithms. The value of score was 0.9 indicates that the *TLR4* proteins are secretary in nature.

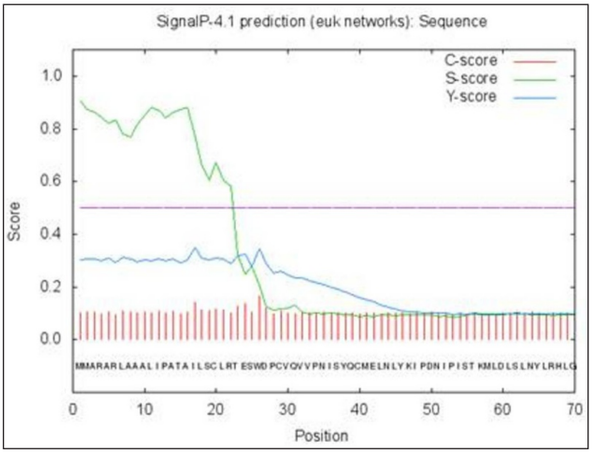

Fig. 2: Signal peptide prediction for *TLR4* protein in Vechur cattle

Leucine and serine were found to be major amino acids in the primary structure of *TLR4* protein. Leucine showed highest percent frequency (16.05 per cent), which is involved in the leucine-rich repeats (LRRs) of ectodomain of *TLR4*, and recognize a particular pathogen through PAMPs. Leucine rich regions recognize various microbial components engaging signaling cascade that results in the response against such microbes (Raja *et al.,* 2011). The second prominent amino acid serine was found to be 8.08 per cent, which is involved in post-translational modification and functional activities.

Amino acid sequence of *TLR4* protein in Vechur cattle was analyzed using SMART (Fig. 3), which revealed regions of ectodomain (1-629), low complexity region (307-327), and cytoplasmic domain (630-819) containing TIR domain (674-819). The ectodomain of *TLR4* displayed different regions, which includes 13 LRRs. There was change in amino acid at LRR5 domain of Vechur when compared to *Bos taurus*. Similarly, 22 LRR domains were reported for *TLR4* gene in goat and also observed variation in the predicted numbers of LRR (Raja *et al.,* 2011). LRR domains are extracellular and involved in identification of PAMPs, whereas cytoplasmic TIR domains are involved in the signal process. The LRR domain is important

Table 4: Multiple sequence alignment for protein sequence of *TLR4* gene

Vechur	MMARARLAAALIPATAILSCLRTESWDPCVQVVPNISYQCMELNLYKIPDNIPISTKMLD	60
Bos	MMARARLAAALIPATAILSCLRTESWDPCVQVVPNISYQCMELNLYKIPDNIPISTKMLD	60
Buffalo	MMARARLAAALIPATAILSCLRTESWDPCVQVVPNISYQCMELNLYKIPDNIPISTKMLD	60
Goat	MMARARRAAALIPAMAILSCLRTESWDPCVQVVPNISYQCMELNLYKIPDNIPVSTEMLD	60
Human	MMSASRLAGTLIPAMAFLSCVRPESWEPCVEVVPNITYQCMELNFYKIPDNLPFSTKNLD	60
	:.:* *.:.** *:***:* ***:***:*****:*******:******:*.**:.**	
Vechur	LSLNYLRHLGSHNFSSFPELQVLDLSRCEIKIIEDDTFQGLDHLSTLILTGNPIQSLAWG	120
Bos	LSFNYLRHLGSHNFSSFPELQVLDLSRCEIKIIEDDTFQGLNHLSTLILTGNPIQSLAWG	120
Buffalo	LSFNYLRHLGSHNFSSFPELQVLDLSRCEIKIIEDDTFQGLNHLSTLILTGNPIQSLAWG	120
Goat	LSFNYLRHLGSHNFSRFPELQVLDLSRCEIKIIEDNTFQGLNHLSTLILTGNPIQSLAWG	120
Human	LSFNPLRHLGSYSFFSFPELQVLDLSRCEIQTIEDGAYQSLSHLSTLILTGNPIQSLALG	120
	:* ****:.* ***************: *** .:*.*.*************** *	
Vechur	AFSGLSSLQKLAAVETNLVSLNDFPIGHLKNLKELNVAHNFIHSFKLPEYFSNLPNLEHL	180
Bos	AFSGLSSLQKLVAVETNLVSLNDFPIGHLKNLKELNVAHNFIHSFKLPEYFSNLPNLEHL	180
Buffalo	AFSGLSSLRKLVAVETNLVSLHDFPIGHLRALKELNVAHNFIHSFKLPEYFSNLPNLEHL	180
Goat	AFSGLSSLQKLVAVETNLVSLDDFPIGHLKTLKELNVAHNFIHSFKLPEYFSNLPNLEHL	180
Human	AFSGLSSLQKLVAVETNLASLENFPIGHLKTLKELNVAHNLIQSFKLPEYFSNLTNLEHL	180
	********:**.******.**.:*******: *********:*:*********** *****	
Vechur	DLSNNKIQNIYYEDVKVLHQMPLLSLSLDLSLNPLDFIEPGTFKEIKLNGLTLRSNFNSS	240
Bos	DLSNNKIQNIYYEDVKVLHQMPLLNLSLDLSLNPLDFIEPGTFKEIKLNGLTLRSNFNSS	240
Buffalo	DLSNNKIQNIYYEDVKVLHQMPLLNLSLALSLNPLDFIEPGTFKEIKLNGLTLRSNFNSS	240
Goat	DLSNNKIQNIYYEDVKVLHQMPLLNLSLDLSLNPLEFIEPGTFKEIKLNGLTLRSNFNSS	240
Human	DLSSNKIQSIYCTDLRVLHQMPLLNLSLDLSLNPMNFIQPGAFKEIRLHKLTLRNNFDSL	240
	.*.** *:.********.*** *****:.**:**:****:*. ****.**:*	
Vechur	HVMTTCIQGLAGLKTNRLVLGEFKNERKLQRFDRSFLEGLCNLTIEQFRIAYLDKFSGDD	300
Bos	HVMKTCIQGLAGLKTNRLVLGEFKNERKLQRFDRSFLEGLCNLTIEQFRIAYLDKFSGDD	300
Buffalo	HVMKTCIQGLAGLKTNRLVLGEFKNERKLQRFDRSFLEGLCNLTIEQFRIAYLDKFSGDD	300
Goat	YVMKTCIQGLAGLKINRLVLGEFKNERKLQKFDRSCLEGLCNLTIEQFRIAYLNKFSRND	300
Human	NVMKTCIQGLAGLEVHRLVLGEFRNEGNLEKFDKSALEGLCNLTIEEFRLAYLDYYLDGI	300
	.*******:.*******:** :*:.**:* **********:**:***:.:	
Vechur	TDLFNCLANVSVISLLSISLGSLQALLKDFRWQHLEIINCDFDKFPALKLSSLKKFVFTD	360
Bos	TDLFNCLANVSVISLLSISLGSLQALLKDFRWQHLEIINCDFDKFPALKLSSLKKFVFTD	360
Buffalo	TDLFNCLANVSVISLLSISLGSLQALLKDFRWQHLEIINCDFDKFPALKLSSLKKFVFTD	360
Goat	TDLFNCLANVSMISLLSIPLGSLQALLKDFRWQHLEMINCDFDKFPALELRSLKKFVFTD	360
Human	IDLFNCLTNVSSFSLVSVTIERVKDFSYNFGWQHLELVNCKFGQFPTLKLKSLKRLTFTS	360
	******:*** :**:*:. : :: : :* *****.:**.* :**:*:* ***:.**.	
Vechur	NKDISTFTEFQLPSLQYLDLKRNHLSFKGCCSHTDFGTTNLKHLDLSFNDVITLGSNFMG	420
Bos	NKDISTFTEFQLPSLQYLDLKRNHLSFKGCCSHTDFGTTNLKHLDLSFNDVITLGSNFMG	420
Buffalo	NKDISTFTEFQLPSLQYLDLKRNHLSFKGCCSHTDFGTTNLKHLDLSFNDVITLGSNFMG	420
Goat	NKDVSSFTKTELPSLQYLDLKRNHLSFKTCCSHTDFGTTNLKHLDLSFNDVITLSSNFMG	420
Human	NKGGNAFSEVDLPSLEFLDLSRNGLSFKGCCSQSDFGTISLKYLDLSFNGVITMSSNFLG	420
	** .:*:: .****::***.** **** ***::**** .**:****** ***:.***:*	
Vechur	LEQLEHLDFQHSTLKQINAFSAFLSLRNLRYLDISYTNIRIVFHGIFTGLVSLQTLKMAG	480
Bos	LEQLEHLDFQHSTLKQINAFSAFLSLRNLRYLDISYTNIRIVFHGIFTGLVSLQTLKMAG	480

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Buffalo   LEQLEHLDLQHSTLKQINAFSAFLSLRNLRYLDISYTNIRIVFHGIFTGLVSLQTLKMAG   480
Goat      LEQLEHLDFQHSTLKQINAFSTFLSLRNLRYLDISYTNIRIVFHGIFTGLVSLQTLKMAG   480
Human     LEQLEHLDFQHSNLKQMSEFSVFLSLRNLIYLDISHTHTRVAFNGIFNGLSSLEVLKMAG   480
          ********:***.***:. **.******* *****:*. *:.*.***.** **:.*****

Vechur    NSFQNNLLPDIFTELTNLTVLDLSKCQLEQVAQTAFHSLSSLQVLNMSHNKLLSLDTFLY   540
Bos       NSFQNNLLPDIFTELTNLTVLDLSKCQLEQVAQTAFHSLSSLQVLNMSHNKLLSLDTFLY   540
Buffalo   NSFQNNLLPDIFTELTNLTVLDLSKCQLEQVAQTAFHSLSSLQVLNMSHNKLLSLDTFLY   540
Goat      NSFQNNLLPDIFTELTNLTILDLSKCQLEQVSWTAFHSLPSLQVLNMSHNKLLSLDTFLY   540
Human     NSFQENFLPDIFTELRNLTFLDLSQCQLEQLSPTAFNSLSSLQVLNMSHNNFFSLDTFPY   540
          ****:*:******** ***.****:*****:: ***.** **********:::***** *

Vechur    EPLHSLRILDCSFNRIMASKEQELQNLPRSLTWLNLTQNAFACVCEHQSFLQWVKDQRQL   600
Bos       EPLHSLRILDCSFNRIMASKEQELQNLPRSLTWLNLTQNAFACVCEHQSFLQWVKDQRQL   600
Buffalo   EPLHSLRILDCSFNRIMASKEQELQNLPRSLTWLNLTQNAFACVCEHQSFLQWVKDQRQL   600
Goat      EPLHSLRILDCSFNRITASKEQELRSLPRNLTWLNLTQNEFACVCEHQSFLQWVKDQRQL   600
Human     KCLNSLQVLDYSLNHIMTSKKQELQHFPSSLAFLNLTQNDFACTCEHQSFLQWIKDQRQL   600
          : *.**::** *:*:* :**:***: :* .*::****** ***.*********:******

Vechur    LVGAEQMMCAEPLDMEDMPVLSFRNATCQLSKTIISVSVVTVLLVSVVGVLVYKFYFHLM   660
Bos       LVGAEQMMCAEPLDMEDMPVLSFRNATCQLSKTIISVSVVTVLLVSVVGVLVYKFYFHLM   660
Buffalo   LVGAEQMMCAEPLDMEDMPVLSFRNATCQLSKTIISVSVVTVLLVSVVGVLVYKFYFHLM   660
Goat      LVGAEQMMCAEPLDVKDMPVLGFRNATCQMSKTIISVSVVTVLLVSVVGVLVYKFYFHLM   660
Human     LVEVERMECATPSDKQGMPVLSL-NITCQMNKTIIGVSVLSVLVVSVVAVLVYKFYFHLM   659
          ** .*:* ** * * : ****.:: * ***:.****.***::**:****.**********

Vechur    LLAGCKKYGRGESTYDAFVIYSSQDEDWVRNELVKNLEEGVPPFQLCLHYRDFIPGVAIA   720
Bos       LLAGCKKYGRGESTYDAFVIYSSQDEDWVRNELVKNLEEGVPPFQLCLHYRDFIPGVAIA   720
Buffalo   LLAGCKKYGRGESTYDAFVIYSSQDEDWVRNELVKNLEEGVPPFQLCLHYRDFIPGVAIA   720
Goat      LLAGCKKYGRGESTYDAFVIYSSQDEAWVRNELVKNLEEGVPPFQLCLHYRDFIPGVAIA   720
Human     LLAGCIKYGRGENIYDAFVIYSSQDEDWVRNELVKNLEEGVPPFQLCLHYRDFIPGVAIA   719
          ***** ******.********** ********************************

Vechur    ANIIQEGFHKSRKVIVVVSQHFIQSRWCIFEYEIAQTWQFLSSRAGIIFIVLQKLEKSLL   780
Bos       ANIIQEGFHKSRKVIVVVSQHFIQSRWCIFEYEIAQTWQFLSSRAGIIFIVLQKLEKSLL   780
Buffalo   ANIIQEGFHKSRKVIVVVSQHFIQSRWCIFEYEIAQTWQFLSSRAGIVFIVLQKLEKSLL   780
Goat      ANIIQEGFHKSRKVIVVVSQHFIQSRWCIFEYEIAQTWQFLSSRAGIIFIVLQKLEKSLL   780
Human     ANIIHEGFHKSRKVIVVVSQHFIQSRWCIFEYEIAQTWQFLSSRAGIIFIVLQKVEKTLL   779
          ****:*********************************************:******:**:**

Vechur    RQQVELYRLLSRNTYLEWEDSVLGRHVFWRRLRKALLAGKPQSPEGTADAETNPQEATTS   840
Bos       RQQVELYRLLSRNTYLEWEDSVLGRHVFWRRLRKALLAGKPQSPEGTADAETNPQEATTS   840
Buffalo   RQQVELYRLLSRNTYLEWEDSVLGRHVFWRRLRKALLAGKPTSPEGTADAETNAQEVTTS   840
Goat      RQQVELYRLLNRNTYLEWEDSVLGRHVFWRRLRKALLAGKPRSPEGTADAETNPQEATTS   840
Human     RQQVELYRLLSRNTYLEWEDSVLGRHIFWRRLRKALLDGKSWNPEGTVGTGCNWQEATSI   839
          **********.**************:********** **  .****.:  * **.*:
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for recognition of specific pathogens. Hence, observed structural variation in LRR domain of *TLR4* might be one of the reasons for high immunity of Vechur cattle against many infectious diseases.

Secondary structure of *TLR4* protein predicted using PSI PRED (Fig. 4), displayed portions of amino acid sequence contributing to formation of α-helix (50.18 per cent), β-turn (17.60 per cent) and random coil (32.22 per cent).

Fig. 3: Predicted domain structure for *TLR4* protien in Vechur cattle

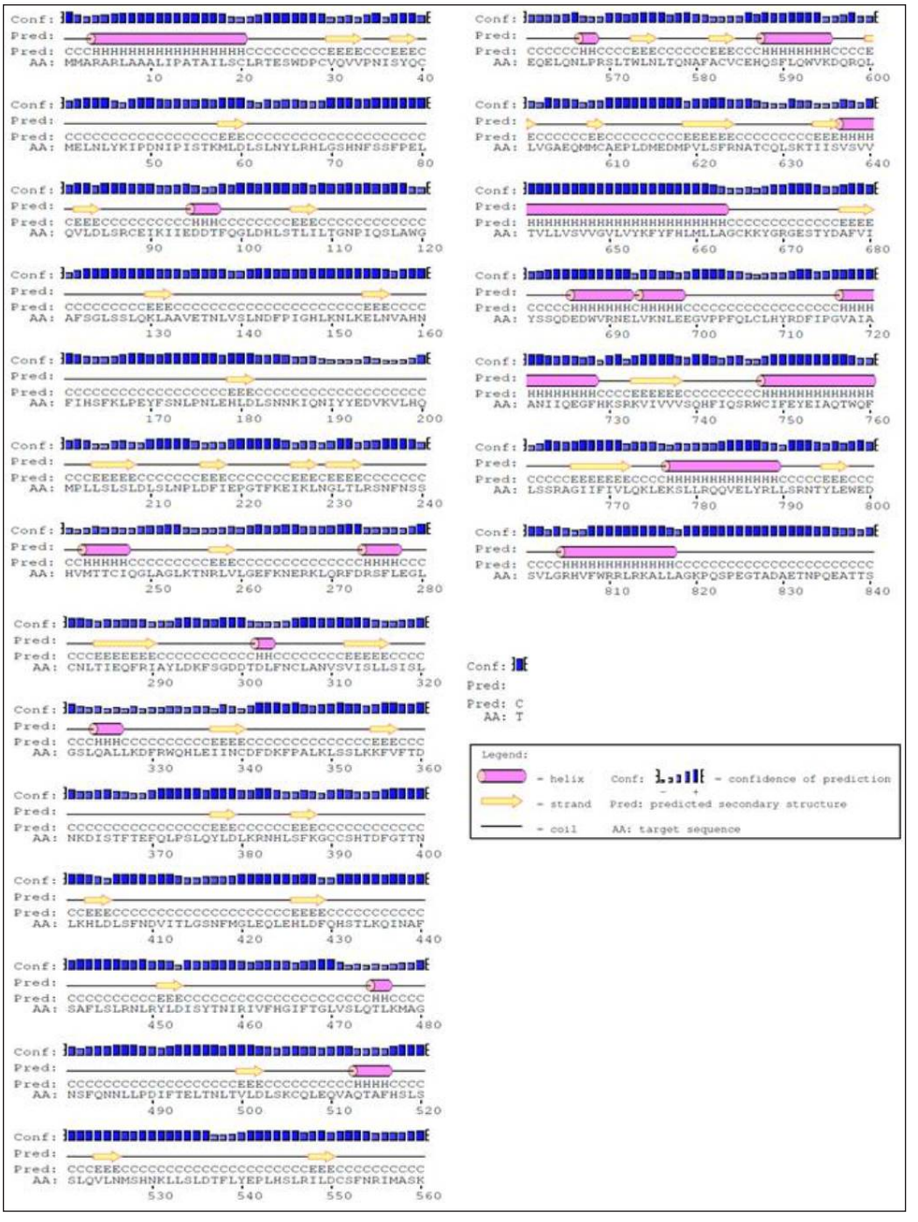

Fig. 4: Secondary structure of *TLR4* protein in Vechur cattle

Similarly, highest per cent of alpha helix for *TLR4* gene was also reported in goat (Goyal, 2012).

Tertiary structure for *TLR4* protein was predicted using SWISS MODEL. The quality score of the tertiary structure of *TLR4* protein had high level of confidence with P-values less than 0.002, whereas the Global model quality scores greater than 0.28. Although the accuracy of our models may still have been limited, the aim of this study was not to describe the tertiary structures precisely but to assess structural similarity between the receptors. The tertiary structure dominated by leucine rich repeats in the shape of a horseshoe, folded together to form a solenoid protein domain. The assembled domain composed of many such repeats, each repeat unit has beta strand-turn-alpha helix structure. The functional domains have been distinguished by different colors (Fig. 5). Ramachandran plot was carried out to visualize backbone dihedral angles ψ against φ of amino acid residues in protein structure. About 99 per cent of amino acid residues are in the favoured and allowed regions that reflects the high stability of predicted protein structure.

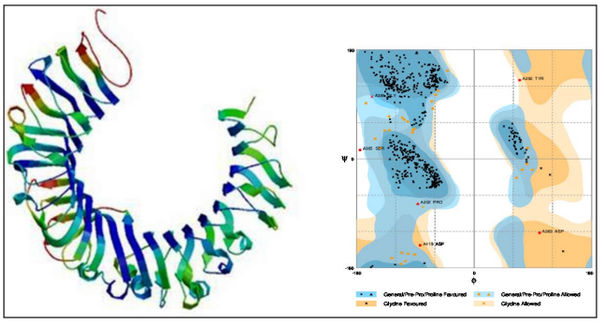

Fig. 5: Tertiary structure of *TLR4* protein with Ramachandran plot

Phylogenetic Analysis for *TLR4* Genes

The nucleotide sequence of Vechur *TLR4* showed high degree identity with *Bos taurus* and *Bos grunniens* (99 per cent) followed with *Cabra hircus* and *Ovis arises* (96 per cent). Protein sequence reveals 99 per cent identity with *Bos taurus* and *Bison bison* and 80 per cent with *Sus scrofa*. *Homo sapiens* were least identical to Vechur cattle, 75 per cent at the nucleotide and 81 per cent at the protein levels.

The phylogenetic tree was constructed based on nucleotide and protein similarities could reveal the evolutionary relationship between various biological species (Fig. 6 and Fig. 7). Phylogenetic tree showed that Vechur cattle was closely related to *Bos taurus*, followed by *Bison bison*, *Bos frontalis*, *Bos mutus* and *Bos grunniens*. *Bos taurus* and *indicus* is much nearer to *Bubalus bubalis* followed by *Ovis aries* and *Capra hircus* thereby grouping the entire *Bovidae* family together (Panigrahi *et al.,* 2014). The results of nucleotide and protein similarity analysis of *TLR4* showed that Vechur cattle fell under *Bovidae* family which indicated that all bovine species come under same ancestor node.

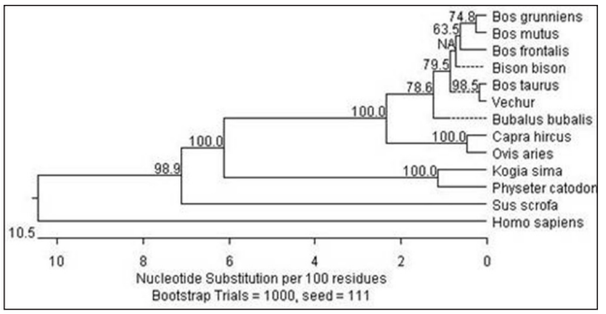

Fig. 6: Phylogenetic tree of *TLR4* with nucleotide sequence

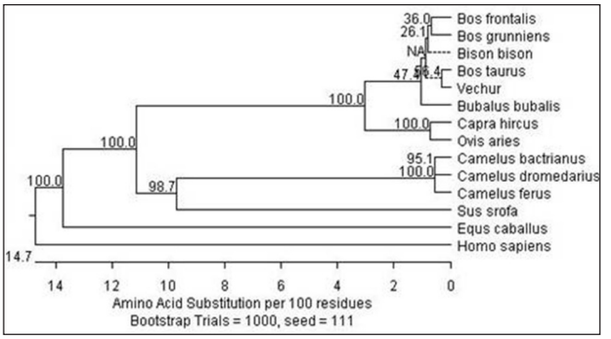

Fig. 7: Phylogenetic tree of *TLR4* with protein sequence

CONCLUSION

To our knowledge, this is the first study to depict sequence and structural variations of *TLR4* in Vechur cattle breed. The observed structural variants in the mRNA sequence of *TLR4* gene in Vechur cattle breed will provide an important basis for further study on the relationship

between polymorphisms and host disease resistance. Data on full-length sequences of the Vechur *TLR4* will be useful for the studies on evolutionary lineages as well as for functional studies for the generation of specific antibodies.

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