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Determination of Genetic Variations in Bovine Toll-Like Receptor 2 Gene in Native Achai and Lohani Cattle Breeds of Khyber Pakhtunkhawa, Pakistan

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AW and TN designed and perceived the experiments. TN, KB and GA executed the experiments. TN, QA, AM and GA analyzed and interpreted the data. QA and TH were involved in sample collection. TN, AM, TH and AW helped in writing and formatted the manuscript. AW supervised the research.

Key words
Toll-like receptors, Variations, Phylogenetic analysis, Achai, Lohani, Khyber Pakhtunkhwa

ABSTRACT

Toll-like receptors (TLRs) are type-I transmembrane pattern recognition receptors (PRRs) that play a critical role in the mammalian innate immune system. They recognize specific molecular patterns from a wide variety of pathogens and initiate a signaling-cascade that mobilizes the appropriate host defense. This study was aimed to determine the genetic pattern in the complete coding sequences of the Toll-like receptor2 (*TLR2*) gene in two agriculturally important indigenous cattle breeds Achai and Lohani of Khyber Pakhtunkhwa (KP) province. Complete *TLR2* gene [5'UTR 136bp, CDS 2355bp, and 3'UTR 1316bp] was sequenced encoding a protein of 784 amino acids long. Out of seven variations observed in CDS of *TLR2* in Achai, 29% were synonymous and 71% were non-synonymous, while in Lohani 27 variations distributed in CDS contained 52% were synonymous and 48% were non-synonymous. Phylogenetic analysis revealed the clustering of both breeds with *Bos indicus* as the nearest neighbor. In both studied breeds, the ratio of dS/dN substitutions was <1 at polymorphic-sites indicating purifying selection. In Lohani cattle, a variation at amino acid position p. Thr174Ile (nucleotide position 521) was presumed to have possible damaging or functional-altering effect. The amino acid sequence analysis revealed signal-peptide followed by an extracellular domain constitute by 20 leucine-rich repeats (LRR), transmembrane and Toll-IL receptor domains. The predicted 3D structure of bovine *TLR2* is a solenoid-like (coil-like) built from 20 LRRs bend into a horseshoe-shaped structure. This study provided an insight into the polymorphisms pattern in *the TLR2* gene that may be potentially associated with PAMPs recognition thus affecting disease susceptibility/resistance animal.

INTRODUCTION

The innate immune system provides an early response to a wide variety of pathogens through germline-encoded cell surface receptors called toll-like receptors (TLRs) (Kloch *et al*., 2018). TLRs are a structurally conserved type-I membrane-bound pathogen recognition receptor (PRR), which is found in both vertebrates and invertebrates (Subhash *et al*., 2018). TLRs play a significant role in the recognition of pathogen-associated molecular patterns (PAMPs) in proteins from viruses, fungi, protozoa, and bacteria and subsequently activated both innate and adaptive immune response mechanisms. Moreover, TLRs also react to host cellular damages called damage-associated molecular patterns (DAMPs) (Tizard, 2009). They are expressed on the cell surface of antigen-presenting cells, such as dendritic cells (DCs), macrophages, T and B cells in bovine (Werling *et al*., 2006). Since the first discovery of a Toll-like protein in the fruit-fly *Drosophila melanogaster*, 10 TLRs (TLR1-TLR10) in bovine, human and chickens and 13 TLRs (TLR1-TLR13) in mice have been reported (McGuire *et al*., 2006). TLRs are

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distinguished by the presence of the Toll-/ interleukin-1 receptor (TIR) domain and LRRs (16 to 28 in numbers) in the extracellular domain involved recognition of pathogens PAMPs (Iqbal *et al*., 2020). All TRLs recognized so far contain three domains despite their amino acid [aa] length, cytoplasmatic Toll/IL-1 receptor (TIR) domain that assists the downstream signal-transduction, a transmembrane domain (TM) which binds signaling molecules and a large extracellular ligand-binding domain (ECD) comprising multiple leucine-rich repeat (LRR) motifs of 20-30 amino acids [aa] which involved in recognition of pathogen's ligands (Bilgen *et al*., 2016).

Bovine *TLR2* gene is mapped to the proximal end of BTA17 and involved in the recognition of bacterial cell components and plays an important role in the immune response against gram-positive bacteria. *TLR2* is forming heterodimers with either *TLR1* or *TLR6* on the plasma membrane and sensitive to several PAMPs like lipopeptides (LP), lipopolysaccharides and teichoic acids (Skevaki *et al*., 2015).

Several past studies have been demonstrated that the polymorphisms in the TLRs genes may diminish the capability of the surface TLRs proteins to recognize the pathogen PAMPs and consequently affect the innate immune activation in mammals (Werling *et al*., 2009). The previous studies have suggested that disease susceptibility and resistance in animals may be caused by single nucleotide polymorphisms (SNPs) that altered ligand binding by TLRs (Dubey *et al*., 2012). Several studies have been performed to identify variations in different breeds of bovine TLR1, 3, 4, 5, 7, 8, 9 and 10 worldwide (Bilgen *et al*., 2016; Cargill and Womack, 2007), however, few studies are performed on especially TLR2 in cattle. Association of polymorphisms in important genes involved in disease resistance in animals may be used as a potential molecular marker for selective breeding.

Achia and Lohani cattle (*Bos indicus*) are draught-purpose breeds distributed in various geographical regions (Swat, Peshawar, and Kohat) of Khyber Pakhtunkhwa (KP) province of Pakistan. Very little information is available on any immunity-related genes in Pakistani cattle breeds and these cattle breeds are not yet characterized for any immunity gene in Pakistan. The key objective of this study was to determine the genetic variations in the bovine *TLR2* gene and it may provide informative genetic markers for future use in association studies of bacterial infection susceptibility or resistance.

MATERIALS AND METHODS

Sample collection

Samples (n= 60) were collected from Achai and Lohani (each breed= 30) from various private and Government livestock farms in districts Swat, Peshawar and Kohat of Khyber Pakhtunkhwa (KP) province of Pakistan. These samples were used to investigate genetic variations in CDS of *TLR2* gene.

Genomic DNA isolation and TLR2 *gene amplification*

Two ml of blood was collected from unrelated animals in EDTA (ethylenediamine tetra-acetic acid) containing vacutainer tubes. The collected tubes were put into ice-containing bags and brought into Animal Genomics Lab, Virtual University of Pakistan (VUP), Lahore. The genomic DNA (gDNA) was isolated with revised phenol-chloroform methods previously described by Wajid *et al*. (2014). The genomic DNA was quantified through Nanodrop spectrophotometry (Nanodrop, 2000c, Thermo Scientific, USA). The complete bovine *TLR2* gene was sequenced using six primer pairs previously used by Subhash *et al*. (2018) (Table I). The *TLR2* gene was amplified in a total reaction mixture of 30µl contained 2.5µl gDNA (20ng), 1µl each forward and reverse primers (10 pmol), 3.5 µl $MgCl_2$ (2.5 mM), 3.5 µl dNTPs (0.25 mM each), 4 µl 1X PCR reaction buffer, 0.5 µl *Taq* DNA polymerase (5 U/µl, Thermo Scientific, USA) and 14 µl DEPC water. The final reaction volume was incubated in Bio-Rad Thermo-cycler with initial denaturation at 95ºC (5 min) followed by 5-cycles at 95°C (30 s), 60°C (30 s), 72°C (30 s) and an additional 30-cycles at 95°C (30 s), 58°C (30 s), 72°C (30 s) with a final extension at 72°C (10 min). The PCR products were run on 1% agarose gel for confirmation and purified using Gen-JET-kit (Thermo Scientific, USA). The purified products were sequenced by automated DNA sequencer (Applied Biosystems, CA, USA).

Table I. Primers used for amplification of *TLR2* gene in the studied cattle breeds.

Genes	Primer Nucleotide sequence 5` → 3`	Size of product (bp)
TLR2-1	F: TCCTGCTCCATATTCCTACG	816
	R: TGACTGTGTTTGACATCATGG	
TLR2-2	F: CTCATTCATTTATGGCTGGC	668
	R: GACCTGAACCAGGAGGATG	
TLR2-3	F: AGATCACCTATGTCGGCAAC	681
	R: CATGGGTACAGTCATCAAACTC	
TLR2-4	F: AGCATCCATCAGTGAAATGAG	774
	R: GGTAAGAAGGAGGCATCTGG	
TLR2-5	F: AGTTTAACCCAGTGCCTTCC	730
	R: TGGAGTCAATGATGTTGTCG	
TLR2-6	F: CCTACTGGGTGGAGAACCTC	436
	R: ACCACCAGACCAAGACTGAC	

Sequencing analysis

The sequences were edited, assembled and analyzed for genetic variations using BioEdit v7 (Hall, 1999). Phylogenetic analysis, sequences percent-identities, and calculation of dN/dS ratio by Nei Gojobori method were performed using MEGA v7 software (Tamura *et al*., 2013). Genetic variations confirmation and positions were retrieved from the ensemble genome browser. LRR finder tool (www.lrrfinder.com) was used for estimation of the location of LRR. Simple modular architecture research tool (SMART) was used to predict the domain structure of *TLR2* protein. PolyPhen-2 (Polymorphism phenotyping v2) software was used for the functional effect of non-synonymous genetic variations. PyMol 2.2.8 was used for tertiary protein structure prediction analysis.

RESULTS

TLR2 *gene sequencing*

The complete *TLR2* gene of 3613 bp (5' UTR 136 bp, CDS 2355 bp and 3' UTR 1316 bp) was obtained from two indigenous Achai and Lohani cattle breeds using six overlapping primer pairs.

Genetic variations in TLR2 *gene of Achai*

The complete CDS of *the TLR2* gene was obtained from Achai cattle by direct sequencing showed 07 variations. Out of 07 variations detected in CDS, 71% (*n*= 5) were non-synonymous at position p.63E>D, p.149Q>P, p.326Q>H, p.345S>N, and p.605M>T and 29% (*n* = 2) were synonymous, which is an average of one variation every 336 bp. Based on the reference sequence from the bovine genome project (ARS-UCD1.2), SNP database at the National Center for Biotechnology Information (NCBI) and published literature, two changes were found to be novel variations: p.149Q>P and p.345S>N (Table II). Of the 07 variations, 29% (n= 2) were C>T, 29% (n= 2) were G>A, 14% (n= 1) were G>T, 14% (n= 1) were A>C and 14% (n= 1) were A>T. Four non-synonymous variations (80%) were detected in the ECD, and one non-synonymous variation (20%) was found in the TM domain. The ratio of dS/dN substitutions was <1 indicating purifying or balancing selection. All the genetic variations were found neutral and have no damaging or functional effects or having benign effects on TLR protein in the studied animals (Fig. 1).

Genetic variations in TLR2 *gene of Lohani*

A total of 27 variations distributed in CDS of Lohani cattle breed, of which 52% (*n* = 14) were synonymous and 48% (*n* = 13) were non-synonymous at position p.125V>A, p.135L>V, p.149Q>P, p.154L>N, p.174T>I, p.248S>N, p.335I>T, p.345S>N, p.527T>F, p.560D>A, p.561D>E, p.563H>R, p.605M>T, p.650R>Q and p.665Q>H. Similarly, when comparing sequences with reference sequence from the bovine genome project (ARS-UCD1.2), SNP database and published literature, 16 changes were found to be novel variations described here are for the first time and the remaining 11 variations have been previously reported (Table III). Out of 27 variations, 45% (n = 12) were C>T, 26% (n = 7) were G>A, 15% (n = 4) were T>G, 7% (n = 2) were G>C and 7% (n = 2) were A>C. A total of 10 non-synonymous variations (77%) were observed in the ECD, and one (8%) and two (15%) non-synonymous variations were detected in TM and TIR domains, respectively. The ratio of dS/dN substitutions was <1 indicating purifying or balancing selection. Out of 13 non-synonymous variations described here, variation at amino acid position p.174T>I (nucleotide position 521) was presumed to have possible damaging or functional altering effect (Fig. 2).

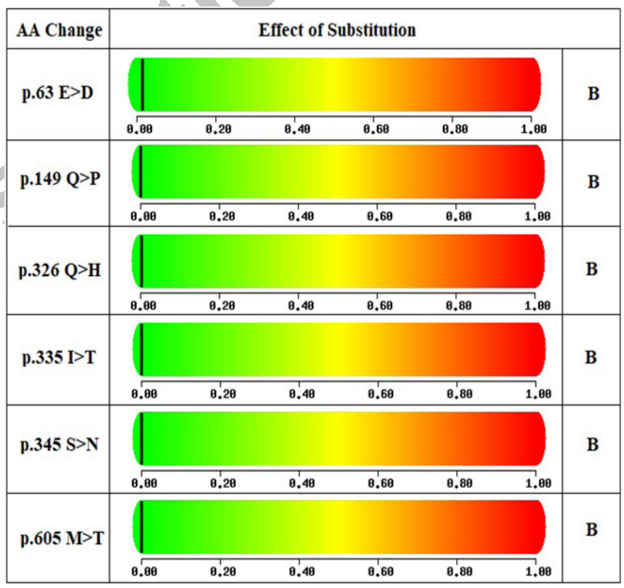

Fig. 1. Effect of amino acid variation in Achai *TRL2*; PD, possibly damaging and B, benign.

Domain prediction of bovine TLR2

Both understudied breeds Achai and Lohani shared similar *TLR2* protein domain architecture, an extracellular domain (ECD), transmembrane (TM) and Toll-Interleukine-I receptor (TIR) domains with predicted molecular weight of 104 kDa and 6.97 pI value. The 20 aa residues of signal peptide were followed by ECD between 54 to 584 aa residues (composed of 20 motifs of LRRs), a TM between 588 to 610 aa residues and TIR domain between 640 to 784 aa residues (Fig. 3a). The secondary structure prediction of bovine *TLR2* protein revealed 45.5% helices, 36% loops and 18.5% β sheets.

Table II. Distribution of genetic variation among the TLR2 gene in Achai cattle.

No.	SNP position	Nucleotide change	Trans/ Transv	Rep/ Nov	AA	AA change	Synonymous/ Nonsynonymous	Protein domain
1	189	G>T	Transversion	Reported	63	E/D	Non-syno	Extracellular
2	446	A>C	Transversion	Novel	149	Q/P	Non-syno	Extracellular
3	978	A>T	Transversion	Reported	326	Q/H	Non-syno	Extracellular
4	1,034	G>A	Transition	Novel	345	S/N	Non-syno	Extracellular
5	1,814	C>T	Transition	Reported	605	M/T	Non-syno	Transmembrane
6	2,214	A>G	Transition	Reported	738	E/E	Syno	TIR
7	2,295	T>C	Transition	Reported	765	P/P	Syno	TIR

Table III. Distribution of genetic variation among the TLR2 gene in Lohani cattle.

No.	SNP position	Nucleotide change	Trans/ Transv	Rep/ Nov	AA position	AA change	Synonymous/ nonsynonymus	Protein domain
1	153	G>A	Transition	Novel	51	T/T	Syno	Extracellular
2	318	T>C	Transition	Novel	106	D/D	Syno	Extracellular
3	320	T>C	Transition	Nobel	107	L/L	Syno	Extracellular
4	374	T>C	Transition	Novel	125	V/A	Non-syno	Extracellular
5	403	T>G	Tranversion	Novel	135	L/V	Non-syno	Extracellular
6	446	A>C	Transersion	Novel	149	Q/P	Non-syno	Extracellular
7	521	C>T	Transition	Novel	174	T/I	Non-syno	Extracellular
8	743	G>A	Transition	Novel	248	S/N	Non-syno	Extracellular
9	750	G>T	Transversion	Novel	250	S/S	Syno	Extracellular
10	801	T>C	Transition	Novel	267	V/V	Syno	Extracellular
11	1,004	T>C	Transition	Reported	335	I/T	Non-syno	Extracellular
12	1,034	G>A	Transition	Novel	345	S/N	Non-syno	Extracellular
13	1,572	A>G	Transition	Novel	524	Q/Q	Syno	Extracellular
14	1,650	A>G	Transition	Novel	550	A/A	Syno	Extracellular
15	1,679	A>C	Trans version	Reported	560	D/A	Non-syno	Extracellular
16	1,683	C>G	Trans version	Reported	561	D/E	Non-syno	Extracellular
17	1,688	A>G	Transition	Reported	563	H/R	Non-syno	Extracellular
18	1,707	C>T	Transition	Reported	569	H/H	Syno	Extracellular
19	1,767	C>T	Transition	Reported	589	A/A	Syno	Transmembrane
20	1,782	T>T	Tran version	Reported	594	A/A	Syno	Transmembrane
21	1,814	T>C	Transition	Reported	605	M/T	Non-syno	Transmembrane
22	1,821	G>T	Trans version	Reported	605	V/V	Syno	Transmembrane
23	1,926	T>C	Heterozygous	Novel	642	D/D	Syno	TIR
24	1,949	G>A	Transition	Novel	650	R/Q	Non-syno	TIR
25	1,995	G>C	Trans version	Reported	665	Q/H	Non-Syno	TIR
26	2,025	C>T	Transition	Reported	675	H/H	Syno	TIR
27	2,055	T>C	Heterozygous	Novel	685	I/I	Syno	TIR

Except for LRR 3 and 13 are formed of purely helical structure, all remaining LRR are found in both sheets and helices (Fig. 3b). Four N-glycosylation sites (at position 114N, 199N, 248N and 442N) were predicted in understudied *TLR2* protein. The predicted *TLR2* extracellular domain based on the homology model Q95LA9/ 5d3iA showed the reliability of the model with a root-mean-square deviation-RMSD value of 0.1A. The eight predicted active site at position Ser 368, Glu 369, Leu 392, Val 393, Leu 409, Thr 411 and Leu 418 forming a pocket for ligand binding in the concave side (Fig. 3b).

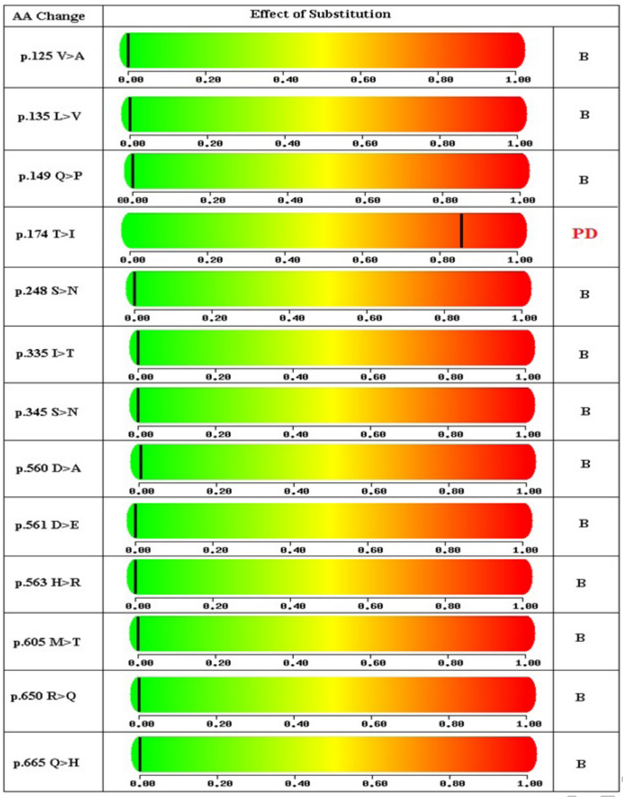

Fig. 2. Effect of amino acid variation in Lohani *TRL2*; PD, possibly damaging and B, benign.

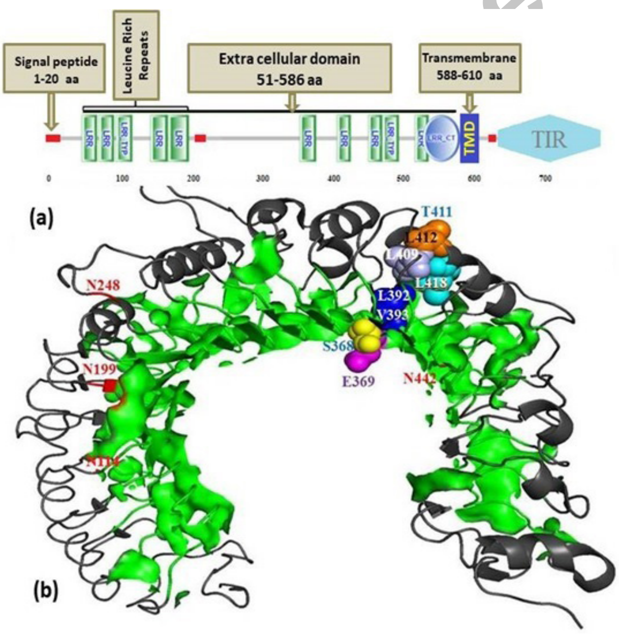

Fig. 3. (a) Domain analysis of 784 aa residues of TLR2 protein (b) Predicted 3-dimensional extracellular domain-architecture of TLR2 protein

Phylogenetic analysis based on TLR2

Phylogenetic analysis was conducted using the Neighbor-Joining method in MEGA v7 software to describe the relationship among mammalian species based on CDS of the *TLR2* gene. The analysis showed close relatedness among mammalian species, i.e. *Bos indicus*, *Bos taurus* were phylogenetically closely related to *Bos grunniens*, *Bos frontalis*, *Bison bison* and *Bubalus bubalis* in a single clade of bovinae. Small ruminants including *Capra hircus* and *Ovis aries* were in a distinct clade with other species *Antidorcas marsupialis*, *Capra ibex* and *Damaliscus pygargus* (Fig. 4). The bovine *TLR2* nucleotide sequences were compared with other mammalian species retrieved from GenBank. High nucleotide sequence similarity 98% to 99% among bovini compared to other ruminants 95% to 97%. High bovine *TLR2* nucleotide sequence dissimilarity (59% to 86%) was observed with horses, dogs, human beings and chickens.

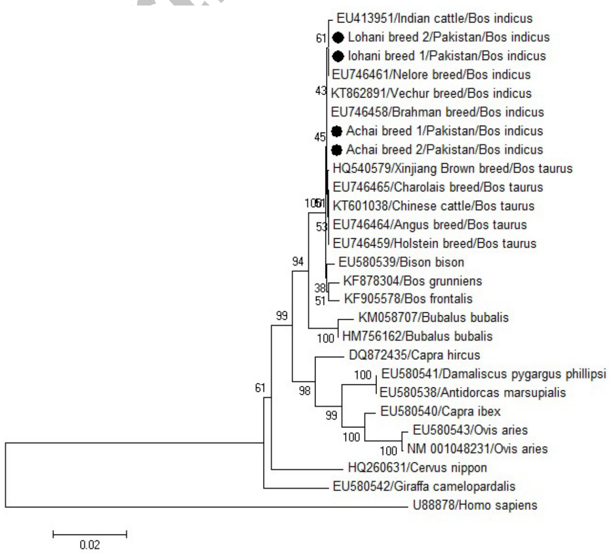

Fig. 4. Phylogenetic analysis was performed based on *TLR2* gene with other species (The studied breeds are indicated with the dark black circle).

DISCUSSION

TLRs are located on cell surfaces characterized as an important class of PRR and play a crucial role in initiating host immune response against foreign invaders (Gay and Gangloff, 2007). TLRs recognize a diverse group of microbial molecules called pathogen-associated molecular patterns (PAMPs) including bacterial-flagellin, lipopeptides, lipopolysaccharide, viral/bacterial ssRNA, viral dsRNA and CpG rich unmethylated-DNA (Akira *et al*., 2006; Subhash *et al*., 2018). *TLR2* mediates cell signaling in response to recognizing a wide variety of bacterial cell components by forming a heterodimer with either *TLR1* or *TLR6* on the plasma membrane. Moreover, heterodimerization of *TLR2* with either *TLR1* or *TLR6* and with non-TLR molecules such as CD-36 expand the repertoire of the ligand spectrum and are critical in the immune response against gram-positive bacteria (Skevaki *et al*., 2015). A genetic mutation in the *TLR2* gene has been associated with disease susceptibility and resistance in several animal species (Iqbal *et al*., 2020). The objective of the present study was to investigate the genetic pattern of *the TLR2* gene in two important cattle breeds Achai and Lohani of KP province, Pakistan. The phylogenetic analysis and nucleotide sequences comparison based on CDS of the *TLR2* gene showed the proximity of ruminant species including cattle, buffalo, goat, sheep with other species gayal, bison and yak (96% to 99%) revealing high conservation of *TLR2* gene. High bovine *TLR2* nucleotide sequence dissimilarity (59% to 86%) was observed with horses, dogs, human beings and chickens. The analysis was consistent with the previous report based on the *TLR2* gene in other Pakistani and Indian breeds (Iqbal *et al*., 2020; Subhash *et al*., 2018). The phylogeny of the *TLR2* gene was consistent with known phylogeny for the ruminant classification based on the mitochondrial cytochrome b (*Cyto b*) gene (Hussain *et al*., 2018), control region (Babar *et al*., 2015) and microsatellite markers (Hussain *et al*., 2016).

Genetic variations previously reported in *the TLR2* gene in animals and humans are likely to be involved in susceptibility to several pathogens. Two mostly studied genetic variations in the *TLR2* gene, a677R>W and 753R>Q have been associated with susceptibility to disease (Kang and Chae, 2001; Lorenz *et al*., 2000). A nonsynonymous SNP i.e. 753R>Q in *TLR2* has been attributed to increasing human predisposition to rheumatic fever (Berdeli *et al*., 2005) and urinary tract infection (Tabel *et al*., 2007) in children, tuberculosis disease (Ogus *et al* 2004) and staphylococcal infection (Lorenz *et al*., 2000). *TLR2* is involved in the early detection of *Mycobacterium avium* subspecies paratuberculosis (MAP) (Quesniaux *et al*., 2004), and past studies showed the association of variations in *TLR2* gene with paratuberculosis (PTB) in bovine (Koets *et al*., 2010; Sadana *et al*., 2015). Moreover, Kumar *et al*. (2019) demonstrated that none of the genetic variations in the bovine *TLR2* gene was significantly associated with the occurrence of PTB in the Indian cattle population. Mucha *et al*. (2009) report a nonsynonymous SNP (220V>M) in the bovine *TLR2* gene has been revealed to decrease the response to MAP. Another study by Koets *et al*. (2010) showed the significant association of -1903T/C (Silent 569) substitution in *TLR2* gene was found to be associated with resistance to MAP infection in cattle, where two prominent genotypes CT and CC were at 1.7-times greater risk than genotype TT for getting MAP infection.

In this study, a total of 5 (71%) and 13 (48%) nonsynonymous variations were observed in Achai and Lohani cattle breeds respectively with a single variation p.174T>I (nucleotide position 521) in Lohani cattle was presumed to have possible damaging or functional altering effects. Moreover, it has been observed that the synonymous mutations can also interfere with gene expression and the 3D structure of *TLR2* (Brest *et al*., 2011). A notable example of synonymous mutation has been documented in the splicing enhancer for the cystic fibrosis transmembrane conductance regulator (CFTR) (Pagani *et al*., 2005). Average frequencies of one substitution in 336 bp and 84 bp were observed in Achai and Lohani cattle, respectively. The average frequencies of substitution in *TLR2* gene in the studied breeds are lower or higher than the previously studied breeds i.e. one per 689 bp in Holstein-Friesian (Koets *et al*., 2010), one per 393 bp in another study of Holstein (Bilgen *et al*., 2016), one per 336 bp in Indian Vulture cattle (Shivakumara *et al*., 2018), one per 168 bp in Tharparkar cattle (Iqbal *et al*., 2020), one per 124 bp in Indian Pahari cattle (Subhash *et al*., 2018), one per 102 bp in Anatolian black, one per 91 bp in Turkish grey, one per 87 bp in East Anatolian red and one per 84 bp in South Anatolian red (Bilgen *et al*., 2016).

In the *TLR2* gene, the 57% and 67% of the variations in Achai and Lohani, respectively fell within the LRR protein domains, the region responsible for ligand binding. The non-synonymous variations located within LRR of ECD might have biological significance for studying the potential association with invading microbes. LRRs are present in bacterial to eukaryotes proteins providing a structural framework for the formation of protein-protein interactions. However, only one variation in TM and two in TIR domains in Achai and four in TM and five in TIR domains in Lohani were detected. This finding is in agreement with the conservation of the TM and TIR domains in murine, human and bovine *TLRs* genes (Pinedo *et al*., 2009; Koets *et al*., 2010).

In both studied cattle breeds, the ratio of dS/dN substitutions was detected <1 indicating purifying selection, the similar selective pressure existed in KP cattle breeds may due to the similar microbial/geographical environment. Both Achai and Lohani are indigenous cattle breeds that evolved under natural-selection over the years. It can be assumed that the genetic variations detected in the present study have a potentially positive effect on immunity traits.

Genetic variations in the *TLR2* gene occur between different cattle breeds and is probably associated with various geographic and therefore pathogen environment. Bacterial infections have been described to cause considerable economic losses in terms of animal production. Genetic variations described here are assumed to be suitable markers for animal screening for susceptibility/ resistance to different bacterial infections. Further studies are required to evaluate the role of these newly identified variations on the immune response and their association with immune-related traits in the animal.

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Ethical approval

This research work on animals was approved by the Departmental Ethical Research Committee of the Virtual University of Pakistan.

Statement of conflict of interest

The authors have declared no conflict of interest.

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