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SHORT COMMUNICATION

Detection of Genetic Variations in Pattern Recognition Receptors (PRRs) Gene of Tharparkar Cattle Breed

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ABSTRACT

The present study was carried out to explore polymorphic patterns in the complete coding sequences of Toll-like receptor2 (*TLR2*) gene in Tharparkar cattle breed originating in Tharparkar district in Sindh province. Complete *TLR2* gene [5'UTR 136bp, CDS 2355bp and 3'UTR 1316bp] was sequenced encoding 784 amino acids. Out of 14 variants observed in CDS, 43% were non-synonymous (NS) and 57% were synonymous (Syn). Phylogenetic analysis revealed clustering of Tharparkar breed with *Bos indicus* as the nearest neighbor. The substitution at amino acid position R to H was assumed to have possible damaging functional effect. The predicted 3D structure of bovine *TLR2* is a solenoid-like structure containing the active sites that form binding pocket for ligand. Thus, our findings suggest that the genetic variations of *TLR2* gene providing important genetic insight into disease resistance in Tharparkar cattle.

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INTRODUCTION

Toll-like receptors (TLRs) are a family of trans-membrane receptors that play critical roles in the innate-immune system by recognizing structurally conserved molecules derived from various microbes. Innate immune system serves as the first line of defense uses a diverse set of pattern recognition receptors (PRRs) to recognize conserved molecular structures known as pathogen or damage associated molecular patterns (PAMPs or DAMPs). Common classes of PAMPs include lipopolysaccharide (cell wall of gram negative bacteria), peptidoglycan (cell wall of gram positive bacteria), polypeptide (e.g. flagellin), and nucleic acids (dsRNA in viruses). PRRs are divided into two main groups including membrane-bound PRRs (TLRs) and cytoplasmic PRRs (NOD-like-receptors [NLRs] and RIG-I-like-receptors [RLRs]) (O'Donovan *et al.,* 2019). Upon Ligand recognition by PRRs, TLRs trigger intracellular-signal pathway that result in the up-regulation of pro-inflammatory cytokines and chemokines. TLRs are the first and highly studied PRRs and are capable of sensing bacterial (*TLR*-1,-2,-4,-5,-6,-9), viral (*TLR*-3,-7,-8,-9) and fungal (*TLR*-2,-4,-6) pathogens (McGuire *et al.,* 2013).

Several previous studies, in both human and animals, have showed that sequence variations in TLR genes influences immune responsiveness, inadequate activation of the innate immune system, weakened recognition of pathogen's PAMP, hereafter interfere with innate-immune activation. The genetic variations in *TLR2* gene may enhance the risk of infectious diseases in human and domestic animals. Several studies have been conducted on distribution of genetic variations for cattle TLRs-1,-3,-4,-5,-7,-8,-9 and -10 and limited reports are available on identification of variations in *TLR2* gene among cattle breeds (Subhash *et al.,* 2018). Describing genetic variations in *TLR2* gene in relation to resistance against particular disease in animal may be used as molecular marker for genetic-selection.

Tharparkar also known as Thari, Zebu-type medium-sized cattle breed originating in Tharparkar district in Sindh province, Pakistan. Dual-purpose breed kept for milk production and also as draft animals. It is dual purpose breed used for milk production and as draft animals. In the

present study, we screened nucleotide sequences of bovine *TLR2* gene to identify genetic variations in Tharparkar cattle breed and the subsequent variations may be used as disease-resistance studies in bovine.

MATERIALS AND METHODS

A total of 49 blood samples were collected from the Tharparkar cattle farm Nabisar Road, Umerkot and local farmers in rural areas of Tendo-Allahyar, Mirpurkhas and Mithi regions of Tharparkar district, Sindh province. All the experiments were performed at laboratory, Virtual University of Pakistan, Lahore. Genomic DNA was extracted using the standard Phenol/chloroform method described by Wajid *et al.* (2014). Complete *TLR2* gene was amplified using six-overlapping primers previously reported by Subhash *et al.* (2018). The reaction mixtures of 25µl containing 1µl of 50 ng/µl DNA-template, 1.5µl of each forward and reverse primer (10pmol), 2.5µl of 10µM dNTPs mix, 2.5µl of $MgCl_2$ (2.5mM), 2µl *Taq* Buffer and *Taq* DNA-polymerase 0.5µl (5unit/µL). The final mixtures were incubated in ABI-thermal cycler at 95°C for 5 mints followed by 5-cycles 95°C, 60°C, and 72°C each for 30s, 30 additional cycles 95ºC, 58ºC and 72ºC each for 30s, followed by last step of 7 mints at 72°C. PCR products were run at 1.3% agarose gel and purified for sequencing using Gen-JET kit (Thermo-Scientific). The sequencing was performed using the automated sequencer ABI 3130XL (ABI, CA). The *TLR2* gene sequences obtained in this study were compared with available sequences of *TLR2* genes retrieved from GenBank. MEGA6 software was used for sequences alignment, and phylogenetic analysis (Tamura *et al.*, 2013). Mutations details of bovine *TLR2* of reference sequence were also retrieved from database Ensemble and compared with our sequencing. LRR-finder tool (www.lrrfinder.com) was used for the estimation of LRRs location, whereas the predicted tertiary protein structure (2Z7X) was generated using the PyMol2.2.8. Simple Modular Architecture Research Tool (SMART) was used prediction of domain architecture.

RESULTS AND DISCUSSION

Toll-like receptors (TLRs) are a family of type-I transmembrane-bound class ofPRRs, responds to evolutionary conserved molecular patterns commonly referred to as pathogen associated molecular patterns (PAMPs). Several relevant studies have been conducted during recent years shown that polymorphisms in the *TLR2* gene can reduce the ability of protein to recognize PAMPs ultimately interfering with the activation of early/ innate immune responses. Functional polymorphisms in TLRs play a significant role in resistance and susceptibility to infectious disease.

In current study, the complete of *TLR2* gene (3613bp) was sequenced and investigated for identification of genetic variations in Tharparkar cattle breed. Open reading frame (ORF) of bovine *TLR2* gene was 2355bp with two-exons encoding 784 amino acids [aa], 136bp long 5'-UTR [untranslated region] and 1122bp long 3'-UTR. Complete coding sequence (CDS) of *TLR2* of Tharparkar showed 14 variations at different positions (Table 1). A total of six variations (43%) were non-synonymous whereas eight variations (57%) were synonymous. Among these variants, 42.8% (n=6) were CT, 14.2% (n=2) were TG, 14.2% (n=2) were TA, 14.2% (n=2) were AG, 7.1% (n=1) were AC and 7.1% (n=1) were GC. However, only one nsSNP was detected in TM and TIR domains seem to be high conserved (Table 1). The synonymous SNPs could also play a role in term of possible regulation of gene expression for *TLR2* (Cargill and Womack, 2007).

Previous studies have demonstrated the influence of genetic polymorphisms in *TLR2* gene on human and animal susceptibility to several pathogens. The R753Q polymorphism in the *TLR2* was associated with tuberculosis, staphylococcal infection, rheumatic fever and urinary tract infection (Subhash *et al.*, 2018) and also other studies reported strong associations of *TLR2* variants with mastitis in cow (Elmaghraby *et al.*, 2018). Another nsSNP (V220M) in *TLR2* gene has been revealed decreased response in bovine to *M. paratuberculosis*, while another *TLR2* SNP (1903T>C) was reported to be associated with resistance to *M. paratuberculosis* infection in cow. Bjelka and Novak, (2020) studied the association of SNP in *TLR2* gene with reproductive traits in Crech Red Pied cattle and reported calf vitality index was correlated with the variant 1044 T>C (rs68268249) in *TLR2* gene. Previous reports revealed the *TLR2* SNPs (L227P, H305P and H326Q) have been associated with immunity related traits in cattle (Bilgen *et al.*, 2016; Shivakumara *et al.*, 2018).

The ratio of dS/dN (ω) substitutions at polymorphic sites was found <1 indicating purifying-selection. An aa at position p.563 out of six non-synonymous variations was assumed to have damaging functional effect, while all other non-synonymous variations having benign effect (Fig. 1). Phylogenetic analysis and nucleotide sequences showed high level of TLR2 gene conservation among *Bos indicus*, *Bos taurus*, *Bubalus bubalis* (>98.5%). High divergence of *TLR2* gene was observed with chicken, dog, horses and human beings (59% to 86%).

Through comparative prediction of deduced *TLR2* aa sequence via SMART revealed three domains including extracellular-EC, trans-membrane and TIR domains (Fig. 2a) related to domain-architecture reported in rabbit, sheep and bovine (Subhash *et al.*, 2018). There were four N-linked glycosylation sites predicted at amino acid positions 114, 199, 248 and 442. The eight predicted active sites at position S368, E369, L392, V393, L409, T411 and L418 forming a ligand binding pocket in the concave side (Fig. 2b). The ER domain contains LRR motifs (different in number in various species) occur in proteins ranging from viruses to eukaryotes providing a structural network for protein with other protein interactions. In this study, a total of three non-synonymous mutations were detected in LRRs of the EC domain of *TLR2* protein that may be changed the receptor recognition of extracellular agents in the LRR domain. The EC domain variations could also advance the PAMPs recognition among pathogens. In the study, the variations detected in *TLR2* gene may be considered as potential molecular markers for functionally significant and immune related traits in bovine. The variants detected in LRR of EC domain may have significant role in investigating the potential associations with pathogens and for regulation of *TLR2* gene expression. The variants reported here may act as potential molecular markers for animal's screening for resistance or susceptibility to various diseases. Further studies are required to fully evaluate any potential associations.

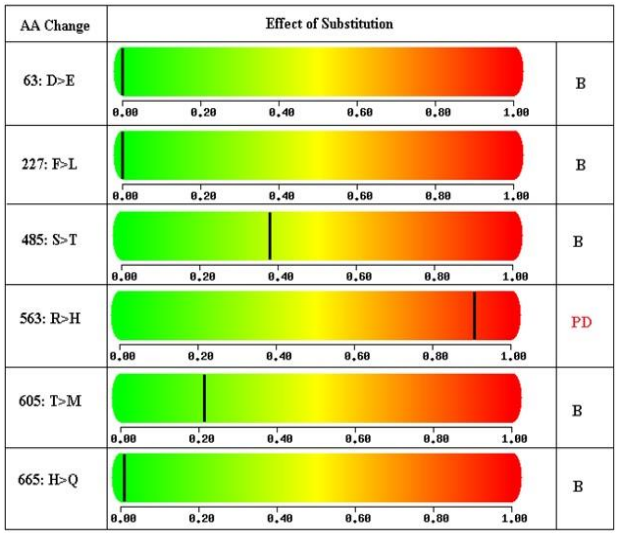

Fig. 1: Effect of amino acid variations of TLR2: B=benign and PD=possibly damaging.

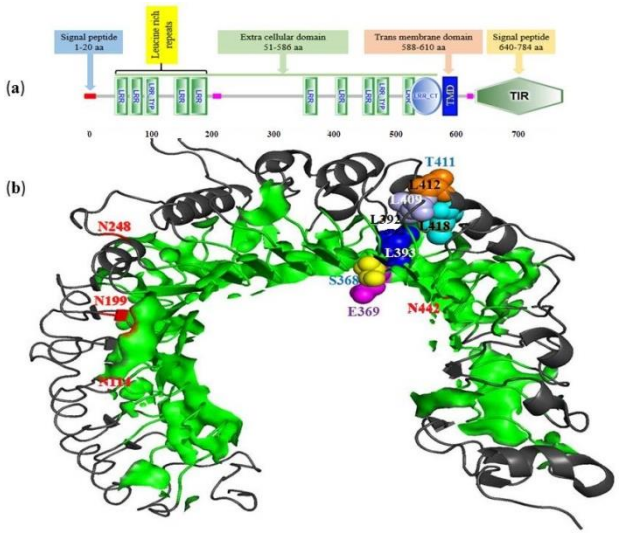

Fig. 2: (a) Domain analysis of bovine *TLR2* (b) Predicted three-dimensional structure of bovine *TLR2*.

Table 1: Synonymous and non-synonymous variations in bovine *TLR2* from Tharparkar cattle

Variant position	Nucleotide change	Transition/ Transversion	AA[a] Position	AA change	Mutation (d_S/d_N)	Protein domain
c.186	T>C	Transition	62	Asn/Asn	dN	Extracellular domain
c.189	T>G	Transversion	63	Asp/Glu[b]	dN	Extracellular domain
c.681	T>A	Transversion	227	Phe/Leu	dN	Extracellular domain
c.1453	T>A	Transversion	485	Gln/His	dN	Extracellular domain
c.1688	G>A	Transition	563	Arg/His	dN	Extracellular domain
c.1707	T>C	Transition	569	His/His	dS	Extracellular domain
c.1779	A>C	Transversion	593	Ala/Ala	dS	Transmembrane domain
c.1782	G>T	Transversion	594	Ala/Ala	dS	Transmembrane domain
c.1814	C>T	Transition	605	Thr/Met	dN	Transmembrane domain
c.1995	C>G	Transversion	665	His/Gln	dN	Toll-Interleukine-1 receptor
c.2025	T>C	Transition	675	His/His	dS	Toll-Interleukine-1 receptor
c.2055	T>C	Transition	685	Ile/Ile	dS	Toll-Interleukine-1 receptor
c.2214	G>A	Transition	738	Glu/Glu	dS	Toll-Interleukine-1 receptor
c.2295	C>T	Transition	765	Pro/Pro	dS	Toll-Interleukine-1 receptor

[a]AA for Amino Acid; [b]Asp/Glu, Aspartic acid changed to Glutamic acid.

Authors contribution: AW, QA, LNI designed and perceived the experiment, LNI, AM, TMY, NH execute the experiment, TH, SS, FM involved in sample collection, AW, LNI, QA, MEB helped in data analysis and write-up.

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