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Characterization of genetic polymorphisms in Toll-like receptor 9 gene of *Bos indicus* Sahiwal cattle

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ABSTRACT

Toll-like receptor 9 protein, located in the endosomal compartment, is a nucleotide–sensing Toll-like receptor (TLR). It is activated by unmethylated cytidine–phosphate-guanosine dinucleotides (CpG ODN) in both viruses and bacteria, and is encoded by Toll-like receptor 9 gene, which was sequenced and characterized in the *Bos indicus* Sahiwal cattle breed. Eleven single nucleotide polymorphisms (SNPs) were detected within the 4.8 Kb region of the TLR9 gene. Eight of the SNPs were present in the coding region of the gene and the other 3 were present in the non-coding part of the gene. The SNP 2930(G>A) was non-synonymous leading to an amino acid change of G437E in the TLR9 protein. The other SNPs were synonymous. These SNPs led to generation of 11 most probable TLR9 gene haplotypes. The gene exhibited a nonsignificant value of Tajima's D which indicated it to be following the neutral mutation hypothesis.

Key words: Genetic variability, Polymorphism, Sahiwal, SNPs, TLR 9

Host defense against invading pathogens including bacteria, fungi, protozoa and viruses is elicited by the immune system which consists of two components, viz. innate immunity and acquired immunity. Recognition of microbial components by TLRs initiates signal transduction pathways, which triggers expression of genes. These gene products control innate immune responses and further instruct the development of antigen–specific acquired immunity (Takeda and Akira 2005, Uematsu and Akira 2006). Toll-like receptors (TLRs) are a class of pathogen recognition receptors that control host immune response through recognition of molecular patterns specific to micro-organisms (PAMPs). They play important role in both innate and adaptive immunity. They are type I transmembrane proteins characterized by an extracellular domain containing leucine-rich repeats (LRRs) and a cytoplasmic tail that contains a conserved region called Toll/ IL-1 receptor (TIR) domain (Werling and Jungi 2003, Jungi *et al.* 2011). So far, 13 TLRs have been identified in mammals of which 10 TLRs are known to occur in cattle, and the expression of TLR transcripts varies among different mammalian species (Lakshmi *et al.* 2016). Among the members of the TLR family, TLR2, TLR4 and TLR9 play an essential role in both innate immunity and adaptive immunity. TLRs are predominantly expressed to various extents in tissues involved in immune function such as spleen and peripheral blood leucocytes, as well as those exposed to the external environment such as lung and gastrointestinal tract. Most of the TLRs are located on the cellular surface with the exception of TLR3, TLR7, TLR8 and TLR9 which are located in the endosomal compartment (Werling and Jungi 2003, Werling *et al.* 2004).

TLR9 (Toll-like receptor 9) is a nucleotide–sensing TLR which is activated by unmethylated cytidine–phosphate-guanosine dinucleotides (CpG ODN) in both viruses and bacteria (Akira and Takeda 2004, Hemmi and Akira 2005, Bowie and Haga 2006, Tanake *et al*. 2010). Toll–like receptor 9 polymorphisms have been observed to be associated with susceptibility to develop meningococcal meningitis in humans (Sanders *et al.* 2012). TLR9 is expressed in the airway epithelium of lungs of cattle, pigs and dogs where it plays an important role in the protection of lungs (Schneberger *et al.* 2011). *M. avium subspecies paratuberculosis* infection dampens CpG ODN responsiveness of TLR9 in cattle (Arsenault *et al.* 2013). Toll-like receptor 9 (TLR9) polymorphism has been reported to be associated with seropositivity of small ruminant Lentivirus in sheep (Sarafidou *et al.* 2013). In studies carried out on water buffalo, the TT genotype at the polymorphic SNP 2340(C>T) was associated with

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susceptibility to tuberculosis (Alfano *et al.* 2014). Because of the role played by TLR9 in a host's defense against viruses and bacteria, TLR9 is a candidate gene for studies of resistance or susceptibility to viral infection in cattle.

Bovine TLR9 is located on BTA22 (McGuire *et al.* 2005). The TLR9 mRNA consists of two exons and is 3255 bp including 5'- and 3'- UTRs according to the NCBI reference sequence (Accession No. NC_007320).

The present study was taken up to characterize the genetic polymorphism present across the genomic sequences of the Toll-like receptor 9 (TLR9) gene of the *Bos indicus* Sahiwal cattle breed by studying the distribution of single nucleotide polymorphisms (SNPs). Sahiwal is one of the best dairy breeds of the Indian subcontinent and also possesses unique adaptability and disease resistance traits like heat tolerance and high resistance to parasites both internal and external. Owing to its distinctive traits of high milk production and heat tolerance, Sahiwal animals have been exported to several other countries of Asia, Africa and Caribbean (Nivsarkar *et al.* 2000, Glass *et al.* 2005, Mason 1996). Single nucleotide polymorphisms (SNPs) are increasingly becoming the marker of choice for investigating contemporary and historical evolutionary genetic processes as they have many advantages over more traditionally used allozymes and microsatellite loci (Allendorf *et al.* 2010, Garvin *et al.* 2010, Slate *et al.* 2010, Seeb *et al.* 2011). These include availability in high numbers, presence in coding and non-coding regions, low scoring error rates, relative ease of calibration between different studies. They are powerful tools to characterize genetic diversity present within and among populations under study (Haynes and Latch 2012).

Although there are previous reports on the molecular structure and polymorphisms present across the bovine TLR9 gene (Cargill and Womack 2007, Griebel *et al.* 2005), so far, to the authors' best knowledge, there has been no study on the SNPs in the TLR9 gene of the *Bos indicus* Sahiwal cattle breed.

MATERIALS AND METHODS

Collection of blood samples: Blood samples (10 ml approximately) were collected from 25 lactating Sahiwal animals. Samples were taken from the jugular vein of the Sahiwal animals (15) reared at Cattle Yard, National Dairy Research Institute, Karnal and similarly from animals (10) at Government Livestock Farm, Hisar in EDTA coated vacuitainer tubes (BD Biosciences). All the necessary precautions and guidelines were followed during blood collection as suggested by the Institute Animal Ethics Committee (IAEC) of NBAGR, Karnal. The animals were not genetically related to each other as was checked from the records at the farms. The animals taken up for this study had genetically unrelated sire(s) and dam(s) and also genetically unrelated grand-sire(s) and grand-dam(s).

Isolation of DNA: DNA isolation was carried out from blood samples by the procedure of digestion with Proteinase K, extraction with phenol: chloroform: isoamylaclohol, followed by precipitation with absolute ethanol (Sambrook *et al.* 1989).

Amplification of the genes by polymerase chain reaction: A set of 10 overlapping primers as specified by Cargill and Womack (2007) (Accession No. NC_007320), were used for the amplification and sequencing of the *Bos indicus* TLR9 gene. The regions of the gene were amplified by polymerase chain reaction following optimal conditions of amplification standardized for each primer pair during the course of the study. Briefly, each 25 µl polymerase chain reaction consisted 50–100 ng of genomic DNA, 2.5 µl of 10× PCR reaction buffer (Sigma), 100 µM each of the dNTPs (Fermentas), 5–10 pmole of each oligonucleotide primer (IDT-USA) and 1 unit of the *Taq* DNA polymerase (Sigma). The PCR was carried out in Mastercycler Gradient from Eppendorf. The thermal cycling profile was: initial denaturation for 2 min at 94°C followed by 30 cycles of denaturation at 92°C for 45 sec, annealing at the optimum annealing temperature for 45 sec and extension at 72°C for 45 sec. Final extension step was for 10 min at 72°C.

Sequencing of the amplified products: The amplified products were purified by QIAquick PCR purification kits and sequenced on ABI 3100 automated DNA sequencer using the Big Dye Terminator dideoxy 3.0, sequencing reaction chemistry. Sequencing was done for both the forward and the reverse primers. In order to avoid artifacts in interpretation and to add to the accuracy of the results, each sequencing reaction was carried out in triplicate.

Statistical analysis: Sequence analysis and alignment was done by Chromas Pro 1.32 (Technelysium Pty. Ltd.), DNA Star (DNASTAR) and Codon Code Aligner (Codon Code Corporation, USA). The normal mode of the Simple Modular Architecture Research Tool (SMART) was used to generate domain architecture depictions of the *Bos indicus* Sahiwal breed TLR9 protein (Schultz *et al.* 1998, Letunic *et al.* 2012). Statistical analysis for generating most probable haplotypes for the gene was done by Phase 2.1.1 (Stephens and Donelly 2003, Stephens *et al.* 2001). Polymorphisms were tested for deviations from Hardy-Weinberg equilibrium by χ^2 – test. Population genetic indices namely gene heterozygosity, gene homozygosity, effective allele number (Ne) were calculated by POPGENE 32 software version 1.32 (Yeh *et al.* 2000). The Tajima's test of neutrality for calculating the D value was performed by using DnaSP v.5 (Librado and Rozas 2009).

RESULTS AND DISCUSSION

The Toll-like receptor 9 gene of the *Bos indicus* Sahiwal cattle breed animals contained a total of 11 SNPs in the 4.8 Kb region. Eight of the SNPs were present in the coding region of the gene and the other 3 were present in the non-coding part of the gene. The SNP 2930(G>A) was non-synonymous leading to an amino acid change of G437E in the TLR9 protein. The other SNPs were synonymous. The numbering of the SNPs positions has been done with respect to FJ495080 which is the TLR9 gene sequence of the first Sahiwal animal studied presently following the rules for

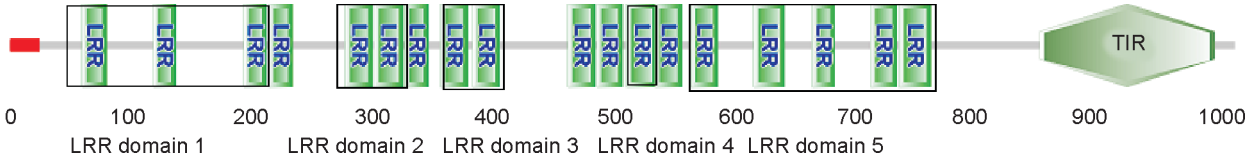

Fig.1. Leucine–rich-repeats (LRRs) domain architecture of the *Bos indicus* Sahiwal cattle breed as generated by SMART. The LRR domains that were observed to be conserved across various species viz. humans, mouse are shown in blocks.

nucleotide numbering according to genomic reference sequence (den Dunnen and Antonarakis 2000). The SNP 2807(G>A) leading to the amino acid change R396H and other synonymous and non-coding SNPs observed in the TLR9 gene of other cattle breeds in previous studies were not found to occur in the *B. indicus* Sahiwal TLR9 gene. Also the SNP V869F and G520R reported to be present in buffalo and sheep was not observed in the present study (Banerjee *et al.* 2011, Sarafidou *et al.* 2013). The SNP 698(C>G) observed in the *B. indicus* Sahiwal TLR9 gene in the present study was not reported in previous studies. The leucine-rich-repeats (LRRs) domain architecture obtained by SMART analysis showed that *Bos indicus* TLR9 protein have 18 LRRs (5 conserved LRR domains) in it which was similar to the domain architecture of the *B. taurus* TLR9 protein (Cargill and Womack 2007) (Fig. 1). LRRs occur in proteins ranging from viruses to eukaryotes and appear to provide a structural framework for the protein-protein interactions.

Two CpG islands were detected in the Sahiwal TLR9 genomic sequence by CpGPlot (http://www.ebi.ac.uk/emboss/cpgplot/) which occur in the TLR9 coding region as in case of previous studies (Cargill and Womack 2007). The first CpG island is located from +3706 to +3964 bp from the first base of the start codon. The second CpG island is located from +4167 to +4587 bp from the first base of the start codon.

Twelve genotypes of the gene were observed in this study (FJ495080, KC174781 –KC174791). The different genotypes along with their frequencies observed in the study are given in Table 1.

Corresponding to these 12 genotypes using PHASE v2.1.1, eleven most probable haplotypes of the TLR9 gene were predicted. The different predicted haplotypes along with their frequencies are given in Table 2.

All the 11 TLR9 SNP(s) were observed to be polymorphic. The observed heterozygosity for almost 73% of SNPs was greater than the expected heterozygosity (0.3132±0.1135) and their F_{IS} value was negative. However, the overall F_{IS} value was very slightly positive (0.0047). F_{IS} is a measure of deviation of the genotypic frequencies from panmictic frequencies in terms of heterozygous deficiency or excess. The value of Ne (the effective number of alleles) was 1.4776±0.2404; the higher the value of Ne, the lesser homozygous are the alleles studied. The genotypes of all SNP(s) except 2916(A>G) were in Hardy Weinberg Equilibrium (Table 3).

To evaluate the spectrum of allele frequencies for single nucleotide polymorphisms, the Tajima's statistic D was calculated (Tajima 1989). The TLR9 gene in the present study showed a Tajima's D value of 0.94627 which was not significant (P>0.10) from 0; which does not exclude the neutral mutation hypothesis, which thereby implies that the 11 segregating sites present across 4.8 Kb length of the gene do not appear to have a biological effect on the functioning of the TLR9 protein of the Sahiwal animals although more studies are required to confirm this.

The present study has yielded information on the various

Table 1. Different genotypes of the TLR9 gene in the Sahiwal animals along with their frequency

Animal (Isolate no.)	NCBI Accession No.	Number of animals	Frequency
Isolate 1	FJ495080	5	0.20
Isolate 2	KC174781	2	0.08
Isolate 3	KC174782	1	0.04
Isolate 4	KC174783	3	0.12
Isolate 5	KC174784	1	0.04
Isolate 6	KC174785	2	0.08
Isolate 7	KC174786	2	0.08
Isolate 8	KC174787	2	0.08
Isolate 9	KC174788	2	0.08
Isolate 10	KC174789	2	0.08
Isolate 11	KC174790	2	0.08
Isolate 12	KC174791	1	0.04
Total		25	1.00

Table 2. Predicted haplotypes (H1 to H11) of the *Bos indicus* TLR9 gene obtained by using Phase 2.1.1

SNP	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11
698(C>G)	G	G	G	G	G	G	G	G	G	G	C
941(T>C)	T	T	T	T	T	T	C	C	C	C	T
1546(T>C)	T	T	T	T	T	C	T	C	C	C	T
1920(C>T)	C	C	C	T	T	T	C	C	C	C	C
2916(A>G)	G	A	A	G	A	A	A	A	A	A	A
2930(A>G)	A	A	G	A	G	G	G	A	A	G	G
3753(C>T)	C	C	C	C	C	C	C	C	C	C	T
4044(A>G)	G	G	G	G	G	A	A	A	A	A	G
4278(A>G)	G	G	G	G	G	G	A	G	G	A	G
4320(C>G)	C	C	C	C	C	C	C	C	G	C	G
4362(A>G)	G	G	G	G	G	G	G	G	A	G	A
Frequency	5	4	9	5	9	2	2	2	3	5	4

Table 3. SNP(s) with their minor allele frequencies (MAF), observed homozygosity, expected heterozygosity, effective allele number (Ne) and F_{IS} observed in the Toll-like receptor 9 gene

SNP	MAF	Observed	Expected homozygosity	Ne heterozygosity	F_{IS}
698(C>G)	0.0800	0.8400	0.1502	1.1726	–0.0870
941(T>C)	0.2400	0.6000	0.3722	1.5743	–0.0965
1546(T>C)	0.2400	0.6000	0.3722	1.5743	–0.0965
1920(C>T)	0.3200	0.3600	0.4441	1.7705	–0.4706
2916(A>G)	0.2000	1.000	0.3265	1.4706	1.000
2930(A>G)	0.3800	0.6400	0.4441	1.8911	0.2360
3753(C>T)	0.0800	0.8400	0.1502	1.1726	–0.0870
4044(A>G)	0.2800	0.5200	0.4114	1.6756	–0.1905
4278(A>G)	0.1400	0.8000	0.2457	1.3172	0.1694
4320(C>G)	0.1400	0.7200	0.2457	1.3172	–0.1628
4362(A>G)	0.1400	0.7200	0.2457	1.3172	–0.1628
Mean ± SD	0.6945±0.1764	0.3132±0.1135	1.4776±0.2404	0.0047	

SNPs present across the TLR9 gene of the *Bos indicus* Sahiwal breed of cattle. The SNP 698(C>G) observed in the intron-1 of the TLR9 gene has so far not been reported in any previous studies of the TLR9 gene in cattle. This SNP may therefore be considered to be a putative novel allele. The information obtained from the present study might be helpful in improving upon the resolution of the genetic markers available across the TLR9 gene for the purpose of mapping and perhaps also for association analysis for the disease resistance traits observed in the Sahiwal animals. The polymorphisms reported here may also assist future evolutionary, genomic and phylogenetic analyses in different bovine breeds.

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