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Phylogenetic and sequence analysis of toll like receptor genes (TLR-2 and TLR-4) in buffaloes

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ABSTRACT

The toll like receptors play a significant role in innate immune response with TLR2 and TLR4 genes being associated with mastitis in cattle. The sequences of these genes were not known in buffaloes and in the present study we sequenced these 2 genes in buffaloes using 24 samples belonging to 6 diverse buffalo breeds of India. Primers were designed from the cattle data base and the buffalo genomic DNA was amplified. The gene sequences obtained after alignment were submitted to NCBI. The TLR2 gene was 3590 bases with 2 exons and coding for 784 amino acids. The TLR4 gene was 4256 nucleotide bases and consisted of 3 exons and coded for 841 amino acids. Eight SNPs were detected in TLR2 gene out of which 3 were non-synonymous. 25 SNPs were detected in TLR4 gene and out of which 10 were non-synonymous leading to change in amino acids. The 2 genes were found to be highly conserved across all mammalian species, and the phylogenetic relationship revealed the closeness of buffalo species with other ruminants, viz. cattle, sheep and goat compared to mono-gastric animals, viz. pigs and man.

Key words: Buffalo, Cattle, Phylogenetic tree, SNP, TLR2 gene, TLR4 gene

Toll like receptors (TLRs), proteins that play a key role in the innate immune system, recognise structurally conserved molecules derived from microbes. Once these microbes have breached physical barriers such as skin or intestinal tract mucosa they are recognised by TLRs which activate immune cell responses. The TLRs play a central role in the initiation of inflammatory responses and subsequent adaptive immune response to microbial pathogens (Sabroe *et al*. 2003,Takeda *et al*. 2003). McGuire *et al*. (2006) recognised 13 TLRs in the mammalian system and mapped 10 of these on bovine genome. TLR2 and TLR4 have been found associated with mastitis in cattle, and play a central role in innate immunity. TLR4 have been found associated with lipo-polysaccharide responsiveness while TLR2 is essential for response to several gram positive pathogens associated molecular patterns (PAMPs) (Schwandner *et al*. 1999, Yoshimura *et al*. 1999). Goldammer *et al*. (2004) suggested that TLR2 and TLR4 may play a role in the host response to inflammatory mastitis.

The two genes were characterized in *Bos taurus* but the sequence of these genes is not available in buffalo. In this paper we present the genomic sequence of these 2 genes in 24 diverse buffalo samples with buffalo specific SNPs.

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MATERIALS AND METHODS

The genomic DNA samples from 24 animals drawn from 6 diverse Indian water buffalo breeds (Murrah, Bhadawari, Tarai, Pandharpuri, Marathwada and Mehsana). The exonic regions were amplified by designing overlapping primers in exon-intron boundaries for both TLR2 and TLR4 genes (Table 1). These primers were designed from sequence available in Bovine Ensembl Transcript for TLR4 (ENSBTAT00000008190) and TLR2 (ENSBTAT00000010530). We simultaneously utilized cattle genomic DNA as a control. 25 µl reaction containing 50ng of sample DNA, 4 pmol of each primer, 0.2 µM of each dNTP, 1u Taq DNA polymerase (Invitrogen) with 1X PCR buffer with 1.5 mM $MgCl_2$ was utilized. Amplification reactions were carried out in GeneAmp with 5 min denaturation at 94°C, 35 cycles of 94°C 45 sec, 57 °C for 45 sec and extension at 72°C for 45 sec followed by a final extension for 10 min. The PCR products were visualized on agarose with 100bp ladder. The PCR products were purified with Exo_SAP enzymatic digestion.

Sequencing was carried out with a final volume of 10 µl using ABI BigDye chemistry with manufacturers' protocol on automated DNA sequencer. Each product was sequenced using reverse and forward primer. The sequences were aligned with SeqScape 2.0. The BLAST was used for

Table 1. The primer pairs used for amplification of genomic DNA

Name	Forward	Reverse
TLR2.1	CTCTgTgCAACCCCAgAgAC	CCACTTgCgTCTACgATTTg
TLR2.2	CAAATCgTAgACgCAAgTgg	CCTACgCCCTCTTCAACACT
TLR2.3	TggAATTAAgCCATgATgTCAA	gTCCgCAgATTTgggAgAT
TLR2.4	CAAATCTgCggACCCTAAAA	TTTAACTCTgCCTgTgAgTggA
TLR2.5	AggCAgAgTTAAAAgAgTCACAA	TCCAACgTCTTCAgTTgCTg
TLR2.6	TCAgCAACTgAAgACgTTgg	CACCACTCgCTCTTCACAAA
TLR2.7	CCACAAAACCATCTTTgTgCT	ATgCCATCCAACCATCTCAT
TLR2.8	gAgggAAggAggAggAgAAg	ggggACACAAAACAgCACTT
TLR4.1	ACTggAACAgACCCTgATgC	ggAAAgCAATCAgACTggTCA
TLR4.2	CCAgTCTgATTgCTTTCCTAAA	gTCCCAACTCTCggTTCTCA
TLR4.3	CAgAgggTCATgCTTTCACA	gggAAACTgAgCAAAgAgCA
TLR4.4	TggAAgTCTgCTAAggTgCAT	ggCTTTCTgACTTgAgAATgAA
TLR4.5	TgggTTgATATTTCATTTTggTT	TTACTTCgCAgAgTCAATCCA
TLR4.6	gCTCAATggATTgACTCTgC	AAgCCCATgAAgTTTgAACC
TLR4.7	ggTTCAAACTTCATgggCTTA	AAgAgCTgCCTCTggTCCTT
TLR4.8	AgTTTCCTgCAgTgggTCAA	TCTgCAggACgATgAAgATg
TLR4.9	CCTgCAgAAgCTggAgAAgT	AggCggTTTCTACTCgAAgg

alignment of sequences of amino acids with other species and genetic distances were utilised for construction of a Neighbor Joining tree.

RESULTS AND DISCUSSION

The 8 primers designed for TLR2 and 9 primers for TLR4 gene (Table 1) from the Ensembl database yielded PCR products of expected sizes. This shows that there is not much difference in these 2 genes between cattle and buffalo and there was complete transferability of primer sequences.

The TLR2 gene sequenced in this study was submitted to NCBI [EU178742]. The sequence was of 3590 bases containing full sequences of exon 1 (54 to 233) and 2 (442 to 2964). This gene codes for 784 amino acids in buffaloes which is similar to other livestock species. However on comparison with *Bos taurus* and *Bos indicus* 18 and 19 changes, respectively, were recorded (supplementry data given on-line). The buffalo sequence was identical to cattle and goat (97%), sheep (92%), and pig (80%). The similarity shows that the changes have occurred during the course of evolution with all the ruminants having more identity among themselves than mono-gastric animals. Eight SNPs were detected in different buffalo breeds of the country. Three of these SNPs were non synonymous (Table 2).

Table 2. Buffalo specific SNPs in TLR2 gene

S.no.	Position	SNP	Change
1	510	C→T*	Threonine 18 Methionine
2	565	C→T	
3	610	G→A	
4	831	T→C*	Valine 125 Alanine
5	976	G→C	
6	2271	T→C	
7	2296	C→T*	Methionine 605 Threonine
8	2383	C→T	

* Non-synonymous.

Table 3. Buffalo specific SNPs in TLR4 gene

S.no.	Position	SNP	Change
1	1341	A→G	
2	1950	A/G→C*	Serine/Tyrosine 191 Cysteine
3	1952	T→C	
4	2025	A→G*	Aspartic acid 216 Glycine
5	2040	A→G*	Aspartic acid 221 Glycine
6	2050	C→A*	Asparagine 224 Lysine
7	2190	G→T*	Arginine 271 Isoleucine
8	2194	A→C*	Leucine 272 Phenlyalanine
9	2275	C→T	
10	3224	G→A	
11	3253	C→T	
12	3266	C→A*	Leucine 630 Methionine
13	3276	C→T*	Threonine 633 Methionine
14	3292	G→A	
15	3295	G→T	
16	3304	G→A	
17	3313	G→A	
18	3325	G→A	
19	3337	C→T	
20	3391	C→T	
21	3415	C→A	
22	3424	G→A	
23	3485	C→G*	Proline 703 Alanine
24	3502	T→C	
25	3842–43	CA→AC*	Glycine 822 Threonine

* Non-synonymous.

The TLR4 gene sequences for buffalo was submitted to

NCBI vide accession number EU386358. The sequence submitted is of 4256 bases with 5' UTR sequence of 914 bases and 3' UTR sequence of 351 bases. The gene consisted of 3 exons (915–1007, 1226–1392 and 1639–3904). The TLR4 gene codes for 841 amino acids and is similar to cattle. The gene is highly conserved with a similarity of >97% with goat and cattle, 94% with sheep, 80% with pig. The total number of SNPs identified among buffalo breeds were 25. Out of these 25 SNPs 10 were non synonymous and resulted in S/T 191 C, A 216 G, A 221 G, A 224 K, L 271 I, L 272 F, L 630 M, T 633 M, P 703 A and G 822 T (Table 3). In non-synonymous SNPs 5 were transitions and rest 5 were transversions.

Thus it is evident that TLR2 and TLR4 genes reveal polymorphism and variations in the susceptibility to mastitis may be attributed to the altered protein structure of both TLR genes. Schmitt *et al*. (2002) demonstrated TLR4 polymorphisms can be used to predict susceptibility to bacterial infection. Growing amounts of data suggest that the ability of certain individuals to respond properly to TLR ligands may be impaired by single nucleotide polymorphisms (SNPs) within TLR genes, resulting in an altered susceptibility to, or course of, infectious or inflammatory disease. Rezazadeh *et al*. (2006) showed an association between genetic polymorphism in TLR4 gene and susceptibility to brucellosis. Sharma *et al.* (2006) reported association of TLR4 SNP with the somatic cell score in Canadian Holsteins. However a direct association between the susceptibility/resistance of mastitis in buffaloes is yet to be established and shall involve association of haplotypes with the phenotypes which were not available for present study.

The phylogenetic analysis clusters *Bubalus bubalis* with the nearest relative *Bos taurus* and *Bos indicus* followed by *Capra hircus* and *Ovis aries*. There is similarity in phylogeny based on both TLR2 and TLR4 genes. Two Neighbour Joining trees constructed on the basis of genetic distances based on the amino acid sequences are depicted in Figs 1,2. Similarity

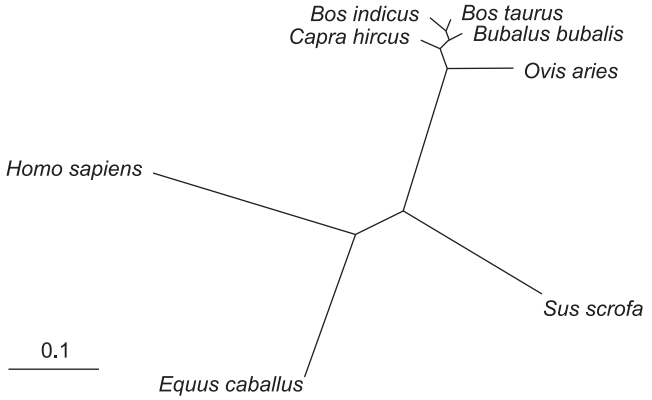

Fig. 1. Neighbour Joining tree of TLR2 protein.

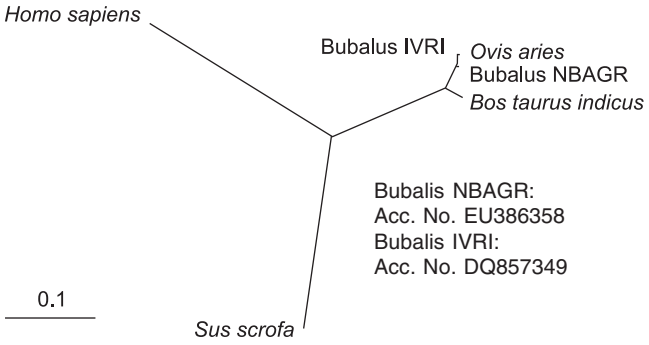

Fig. 2. Neighbour joining tree of TLR4 protein.

of these gene sequences in ruminants points to high level conservation of these genes/proteins which are associated with innate immune response. There is emerging global interest in genomic selection in beef and dairy cattle, where evidence for balancing selection on at least 2 of the TLR genes (TLR3 and TLR8), with detection of a weaker selective signal consistent with purifying selection in TLR10 (Seabury *et al.* 2007). TLR3 and TLR8 encode molecular sentries that recognize invading double-stranded (ds) and single-stranded (ss) RNA viruses, respectively, thereafter eliciting host innate immune responses (Akira and Takeda2004, West *et al.* 2006). The selection on TLR3 and TLR8 can have direct implications on aspects of differential susceptibility to major viral production diseases such as blue tongue (dsRNA, Reoviridae), foot and mouth disease (ssRNA, Picornaviridae), bovine viral diarrhea (ssRNA, Flaviviridae); calf coronavirus (ssRNA, neonatal diarrhea, Coronaviridae), and bovine parainfluenza 3 (ssRNA, Paramyxoviridae) (Fauquet *et al.* 2005, Cahn and Line2005). Moreover, evolution under repeated exposure to many of these diseases may provide some explanation for the observed patterns of variation detected within TLR3 and TLR8. However it is also possible that more ancient host-pathogen interactions (i.e., eradicated Rinderpest, ssRNA, Paramyxoviridae; etc) may have contributed to the signatures of selection detected for cattle (Fisher *et al.* 2011). Future studies involving all species of the subfamily Bovinae which include the buffalo are needed to help elucidate whether selective signals in TLRs extend beyond modern domestic cattle lineages. The variation within TLR genes needs comprehensive evaluation with respect to differences in ligand-induced signaling, disease susceptibility, and the potential for marker-assisted vaccination in domestic cattle or for selection in buffaloes. In addition to selective signals observed for TLR3 and TLR8, several tentative associations were detected between bovine TLR SNPs (Fisher *et al.* 2011). Previous studies (Hasan *et al.* 2005 and Guan *et al*. 2010) indicated that human TLR10 forms functional heterodimers with both TLR2 and TLR1, thereby enabling the resulting protein complexes to recognize a wide variety of microbial ligands (Guan *et al.* 2010), including those derived from *Mycobacteria* (Uciechowski

et al. 2011). Similarly, TLR2 is also known to form functional heterodimers with TLR6 (Ozinsky *et al.* 2000). Recently, AA substitutions in human TLR1 and TLR10 were demonstrated to negatively impact receptor function (Guan *et al.* 2010 and Uciechowski *et al*. 2011), with TLR10 ligand recognition similar to the known range of ligands established for TLR1 (Uciechowski *et al*. 2011). The results of single marker association tests indirectly support the biological concept of functional unity with respect to bovine TLR2, TLR6, and TLR10, with variation at all three loci categorically linked to a common microbial phenotype in Holstein cattle (Fisher *et al*. 2011). TLR2 and TLR4 are the genes associated with mastitis and cause huge economic losses and to understand the underlying mechanism of these two TLRs is of paramount importance.

A large number of variations observed in the buffalo populations make these genes likely candidates for variations observed at the phenotypic level attributed to differences in the susceptibility /resistance to various bacterial infections. More so these variations can act as markers for various epidemiologic studies as well as diversity analysis in buffaloes.

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