



Sequence and phylogenetic analysis of Toll like receptor genes TLR-3 and TLR-9 in buffaloes

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ABSTRACT

Toll like receptors (TLRs) play an important role in the innate immune recognition. Ten members of the TLR family have been identified in bovine and 2 of these recognize PAMPs specific to viruses (TLR3 and TLR9). The main objective of this work was to sequence the 2 genes in *Bubalus bubalis*, detection of SNPs in these 2 genes in different buffalo breeds, prepare gene tree and compare amino acid sequences with other species for construction of phylogenetic tree. The primers for the 2 genes were designed from the cattle genome database. The genomic DNA samples from 24 animals drawn from 6 diverse breeds of Indian water buffalo (Bhadawari, Kerela, Murrah, Pandharpuri, Surti and Tarai) were utilized. The amplified genomic DNA yielded fragments of expected sizes. The alignment of sequences with *Bos taurus* as reference sequence, permitted the comparative analysis of buffalo TLR3 and TLR9 gene and the presumed protein sequence with respect to cattle. TLR3 consisted of 5 exons in which 6 SNPs were detected, out of which 4 were non-synonymous whereas, TLR9 comprised of 2 exons and 7 SNPs were identified, out of which 1 was non-synonymous. These SNPs in the exonic regions were novel and has potential for their use in diversity analysis as well as association studies with tolerance to bacterial and viral infections in buffaloes. The phylogenetic analysis revealed even-toed animals joining first followed by other mammals which later joined by poultry species. The last to join the tree were bony fishes (Sarcopterygii). Surprisingly, TLR9 showed similarity with TLR7 in all the species studied for which information is available in the database. This has been reflected in the phylogenetic tree.

Key words: *Bubalus bubalis*, Cattle, Innate immunity, Single nucleotide polymorphism (SNP), Toll-Like receptors

Vertebrates have developed a system of immune-defense to counteract microorganisms. This system forms the first line of host defense mediated by phagocytes including macrophages and dendritic cells. The immune system recognizes microorganisms via a number of germline-encoded pattern recognition receptors (PRRs). These PRRs recognize microbial components known as PAMPs (pathogen associated molecular patterns) essential for survival of microorganisms and difficult for microorganisms to alter. Several classes of PRRs, including Toll-like receptors and cytoplasmic receptors, recognize distinct microbial components, which activate immune cells by responding to endogenous molecules released as a consequence of cellular damage (Akira and Takeda 2004). Indian dairy industry loses ₹ 60 billion due to infection of the udder. The genetic causes of mastitis are not precisely known, and can best be

studied by investigations of various genes affecting the trait and variations underlying them.

At least 10 members of TLR are identified in bovines and 13 in mice. In the present investigations, we concentrated on TLR3 and TLR9 which are located on chromosomes 27 and 22 respectively. TLR3 responds to double stranded RNA in virus and TLR9 responds to unmethylated CpG DNA in bacteria as well as virus (Bowie and Haga 2006). The recognition of viral components by PRRs commonly induces Type I interferon (IFN) production. Double stranded RNA along with its synthetic analog, polyinosinic:polycytidylic acid (Poly I:C) is a potent inducer of Type I IFN recognized by TLR3 (Alexopoulou *et al.* 2001). Bacterial genomic DNA is an immuno-stimulant due to unmethylated CpG dinucleotide in a particular base context which is recognized by TLR9 (Hemmi *et al.* 2000 and Krieg 2002). They are also involved in the recognition of viral nucleotides rich in CpG – DNA motifs by PRR which induces type I IFN production that activate target cells (Lund *et al.* 2003, Hochrein *et al.* 2004, Tabeta *et al.* 2004, Krug *et al.* 2004a, 2004b). Toll-like receptors bind and become activated by different ligands,

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which in turn are located on different types of organisms or structures. They also have different adapters to respond to activation and are located sometimes at the cell surface and sometimes in the internal cell compartments.

Thus this study was undertaken to characterize TLR3 and TLR9 genes in *Bubalus bubalis* and detection of SNPs in these 2 genes in different buffalo breeds, preparation of gene tree and comparing the amino acid sequences with the near relatives of buffaloes.

MATERIALS AND METHODS

The genomic DNA samples from 24 animals drawn from 6 diverse breeds of Indian water buffalo (Bhadawari, Kerela, Murrah, Pandharpuri, Surti, and Tarai) were taken. Bhadawari breed belongs to ravines and is adapted to extensive production while Tarai and Murrah are North Indian buffaloes, Pandharpuri and Surti belong to Western regions.

The exonic regions were amplified by designing primers in exon-intron boundaries for TLR3 and TLR9 genes. These primers were designed from sequence available in Bovine Ensembl Transcript for TLR3 (ENSBTA00000011445) and TLR9 (ENSBTAT00000024223). All PCR primers were designed using web interface of Primer3. PCR primers for amplification of both the TLR genes are given in Table 1. Cattle genomic DNA was used as a control. The reaction volume of 25 µl containing 50ng of template DNA, 5 pmol of each primer, 0.2 µmol of each dNTP, 1 unit of Taq DNA polymerase with 1 X PCR buffer and 1.5 mM MgCl₂ was utilized. Amplification reactions were carried out in ABI 9700 Gene Amp with a program as 5 min denaturation at 94°C followed by 35 cycles of 94°C for 45s, annealing at 57°C for 45s and extension at 72°C for 45s, with a final extension of 10 min at 72°C. The PCR products were resolved on 2% agarose gel with 100bp ladder. Prior to sequencing the PCR products were treated with Exo-SAP. Sequencing was carried

out with a final volume of 10 µl with manufacturer's protocol on automated DNA sequencer. Each primer was sequenced using reverse and forward primer. The sequences were aligned using SeqScape 2.0. The sequences were translated to get respective amino acid sequences for the genes. These amino acid sequences were aligned using CLUSTALW multiple alignment in BIOEDIT. The phylogenetic trees were prepared using BLAST to compare the change in amino acid sequences with other members of family *Bovinae*.

RESULTS AND DISCUSSIONS

The amplified PCR products were of expected sizes. The sequences were aligned with one another to yield the consensus sequence of complete genes. The sequence of TLR3 gene was submitted to NCBI (FJ606788). The TLR3 consisted of 5 exons and our sequence consisted of 4355 bases. The 5'UTR region in the present study was of 109 bases while 3'UTR was 196 bases long. The TLR3 gene codes for a protein of 904 amino acids. The protein Id for the gene is ACM68434.2. The TLR3 gene has conserved domains and belongs to TIR superfamily. The gene shares a similar signaling pathway with Interleukin-I-receptor. Six SNPs were detected in TLR3 gene with 24 diverse buffalo samples. Out of the 6 SNPs detected, 4 were transversions while only 2 were transitions; these 4 were non-synonymous. The nucleotide position with respect to accession no. FJ606788 and the change in amino acid are given in Table 2.

The sequence of TLR9 gene consisted of 3598 nucleotide bases (FJ606787). The gene consists of 2 exons. 5'UTR of 226 bases and 3'UTR of 29 bases was also sequenced. The first exon is small and codes for a single amino acid. The second exon is from 483 to 3569. The gene codes for a total of 1029 amino acids. This TLR also belongs to TIR superfamily. A total of 7 SNPs were found in the exonic regions out of which only one SNP was non-synonymous.

Table 1. The primer pairs used for amplification of exons for TLR3 and TLR9

Primer	5'→3' Forward primer	5'→3' Reverse primer
<i>TLR3</i>		
3_01	CATGGAAAAGGAAAAAGAAGACA	CCACTTCAAGCACCAGATCA
3_02	CACATAGTCACGTTTGTTGC	TGATGCATAAACGACTTGAAAA
3_03	AGATGTGCTATTTTGATGTCCA	CCAGCGAGCGATAGAAATGT
3_04	GTGCCTGTTTTCCAAAGGTC	CCAGCGAGCGATAGAAATGT
3_05	CATTTCTATCGCTCGCTGGT	TCAAAGACAGATTGTGGAAGGA
3_06	TCCTTCCACAATCTGTCTTTGA	ACTGCCGCAACTAAACTGG
3_07	AACCTGGGTTTTAGTGACAGGA	TCACAAACCCTCATATACCTCTAACTT
<i>TLR9</i>		
9_01	GCGATCTGCCACCTGACTAT	CCCACCCTTGTCTGTAGCTC
9_02	AGGTGAGGCAGAGGAGGTG	CACCTCCGTGAGGTTGTTGT
9_03	CGCTCAAGTACAACAACCTCA	GACAGGTCCAAGAGCAG
9_04	GAGCCTGCTCTTCGTGGA	ACCAGGTTCTCAAGCCTTT
9_05	ACCTGTCCGAGAACCATCTG	CACAGGAAGCAGTACCA
9_06	TGGGACCTCTGGTACTGCTT	TTAAAGGAGTGCTGGGCAGT

Table 2. Buffalo-specific SNPs in TLR3 gene

Position	SNP	Amino acid position
2157	A/T*	I347N
2769	G/A*	S551N
3019	A/G	
3064	A/T*	E649D
3163	C/A	
3593	C/G*	V826L

Table 3. Buffalo-specific SNPs in TLR9 gene

Position	SNP	Amino acid position
193	T/C	
2741	C/T	
2771	C/T	
2819	C/T	
2852	A/G	
2954	A/G	
3084	G/T*	V869F

* Non-synonymous for Tables 2 and Table 3.

The nucleotide change at 3084 was a transversion and leads to change from valine to phenylalanine (V869F) as given in Table 3.

The nucleotide sequences of 2 genes were utilized using

neighbour-Joining algorithm (Saitou and Nei 1987) to construct a tree using BLAST (Altschul *et al.* 1990) and NCBI database. The branch of even-toed ungulates were analysed in detail. The nucleotide sequences for TLR3 genes were compared to other species using BLAST and a neighbour-joining tree was constructed. The present study was restricted to even-toed ungulates. The sequence obtained in this study was similar to other sequences available in the NCBI database of *Bubalus bubalis*. They had similarities with *Boselaphus tragocamelus*. The group joined another group of sequence from *Bos taurus* and *Bos indicus* followed by *Ovis aries* for TLR3 gene. All these sequences joined another group of sequence belonging to a *Sus scrofa* (Fig. 1).

The nucleotide sequence of TLR9 deposited by us matched the other sequences already deposited in the NCBI GenBank of *Bubalus bubalis*. The next similar sequences belonged to *Bos taurus* and *Bos indicus* sequences followed by sequences of the species *Boselaphus tragocamelus* for TLR9 sequence. The group of sequences joined the sequence of *Capra hircus* and *Ovis aries*. The 2 groups later joined with *Antelope cervicapra* sequence of TLR9 (Fig. 2).

The nucleotide sequences of TLR3 and TLR9 were translated into amino acids. The amino acid sequences of the 2 genes were analysed for similarities between different species. There was a great degree of similarities among different mammalian species. The amino acid sequences obtained in this study for TLR3 and TLR9 were utilized for

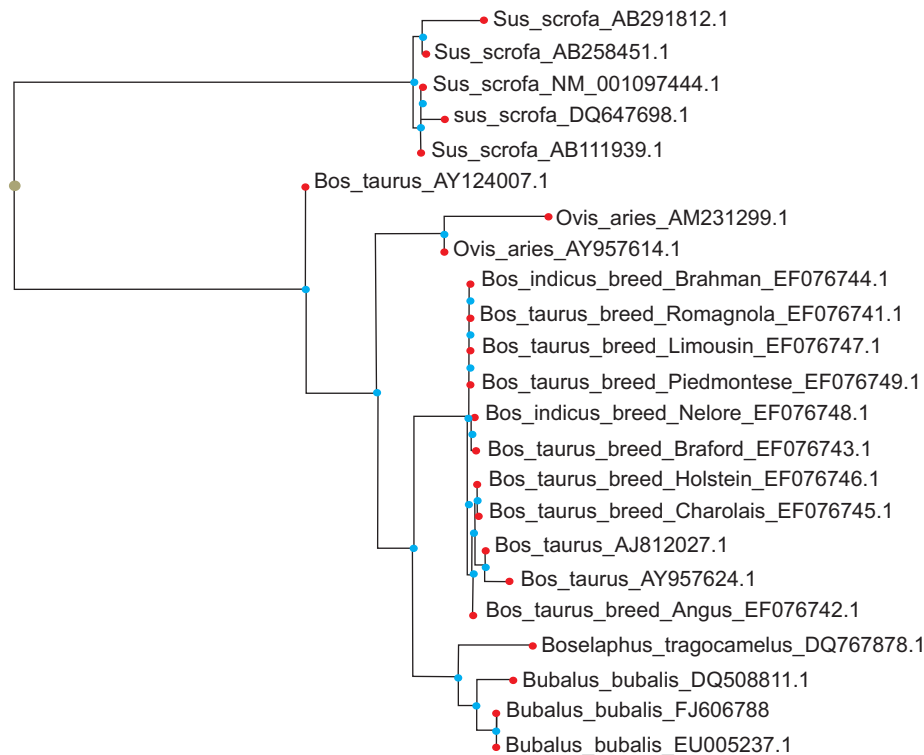


Fig. 1. Phylogenetic tree of TLR3 based on nucleotide sequences.

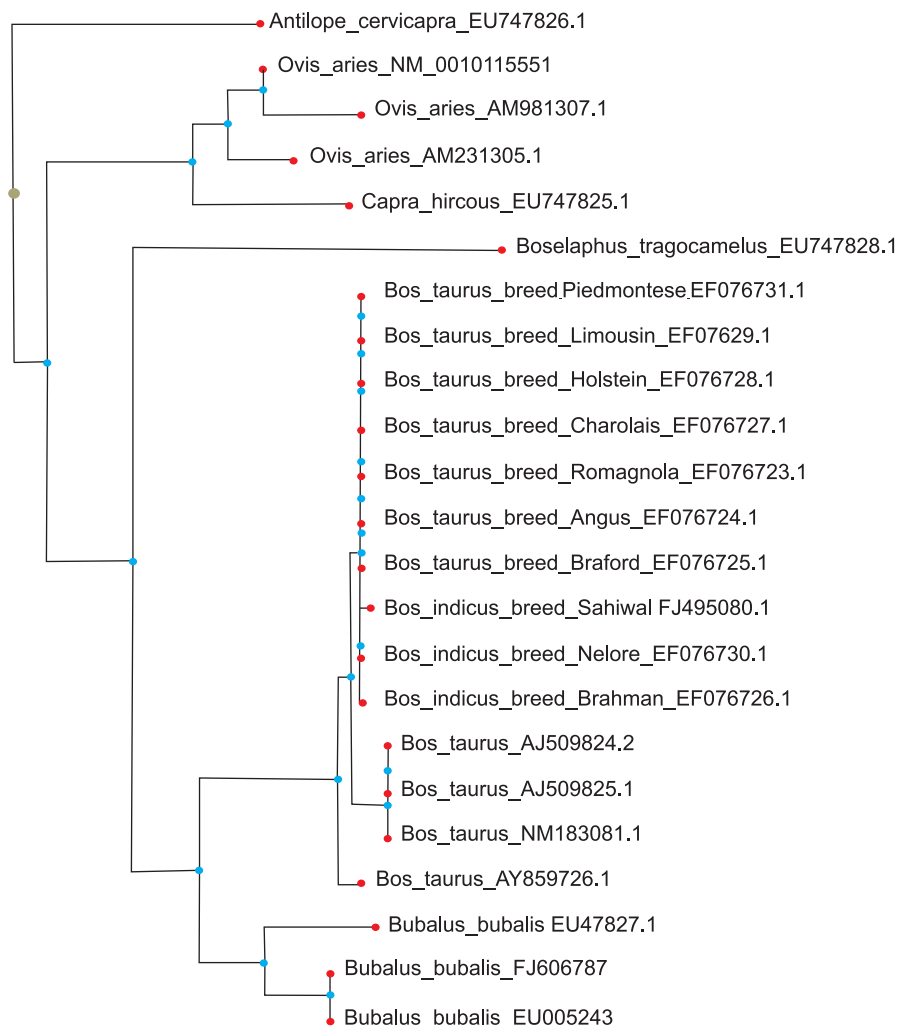


Fig. 2. Phylogenetic tree of TLR9 based on nucleotide sequences.

BLASTp. The neighbour joining tree was constructed for the 2 genes and is depicted in Figs 3 and 4. The BLASTp of TLR3 revealed very similar results in the evolution with mammals joining the birds (*Gallus* sp.) followed by members of *Sarcopterygii*. The overall divergence among all species was 9% for TLR3 (Fig. 3).

The Fig. 4 for TLR9 reveals similarity with *Bos taurus* and *Bos indicus* amino acid sequences. This is expected amongst all large ruminants. The tree is joined by small ruminant like *Capra hircus* and *Ovis aries* followed by other livestock species. Equines and other domestic animals like cats, dogs join the phylogenetic tree next. This larger group joins other mammalian species like *homo sapiens*, chimpanzee, gorilla followed by monkeys. Rats and mouse form a separate cluster and join the human group. The BLAST surprisingly showed striking similarities with TLR7 producing a separate group of *Bos taurus*, *Ovis aries*, *Canis lupus*, *Equus caballus*. These 2 groups are joined with members of *Sarcopterygii* which are bony fishes with flesh. The overall

divergence among species was 10% for TLR9 gene.

The comparison of buffalo with *Bos taurus*, *Bos indicus*, *Ovis aries*, *Sus scrofa*, *Equus caballus* and *Homo sapiens* was carried out using CLUSTALW alignment for TLR3 amino acid consisting of 904 amino acids. The alignment revealed change at position 13 where buffalo has threonine compared to serine in *Bos indicus* and *Bos Taurus*, which seems to be highly variable. Similarly at location 221, 257, 326, 327, 347, 497, 522, 566, 818 and 904 amino acids were different compared to other species with which the comparison was made. There were few instances where the amino acids were similar to other species but differed from cattle (*Bos indicus* and *Bos taurus*). The variable sites were 192, 221, 223, 563 and 818.

Similarly, the gene TLR9 seems to be highly conserved across the species. The CLUSTALW alignment of the amino acid sequences revealed only 14 changes. The amino acid 22 seems to be variable among the species. The amino acid 44 which is asparagine in buffalo is similar to other species



Fig. 3. Phylogenetic tree of TLR3 based on amino acid sequences.

but different to cattle where it is aspartic acid. Similar changes at amino acid 111, 152, 159, 181, 217, 308, 362, 637, 659, 859 and 934 were seen.

The phylogenetic tree constructed on the basis of nucleotide sequences for TLR3 revealed closeness of buffalo to cattle (*Bos indicus* and *Bos taurus* on first node) followed by *Ovis aries* and then by *Sus scrofa*. Similarly phylogeny is revealed for TLR9 gene in cattle.

The nucleotide sequences for TLR3 and TLR9 gene are the first report in *Bubalus bubalis* at genomic DNA level. The analysis revealed that these 2 genes are highly conserved

across all the mammalian species. There has been only 1 non-synonymous SNP in the diverse buffalo population study. The change was valine to phenylalanine at nucleotide position 3084. The nucleotide sequence of TLR3 is associated with viral infection and is thus highly variable. The nucleotide data revealed the diversity among different buffalo populations in India. There were as many as 4 changes in the amino acid sequences of the buffalo population in India. These may be due to large diversity in viral genome and TLR3 is likely to be involved in early pathogen detection of host response to viral infection.

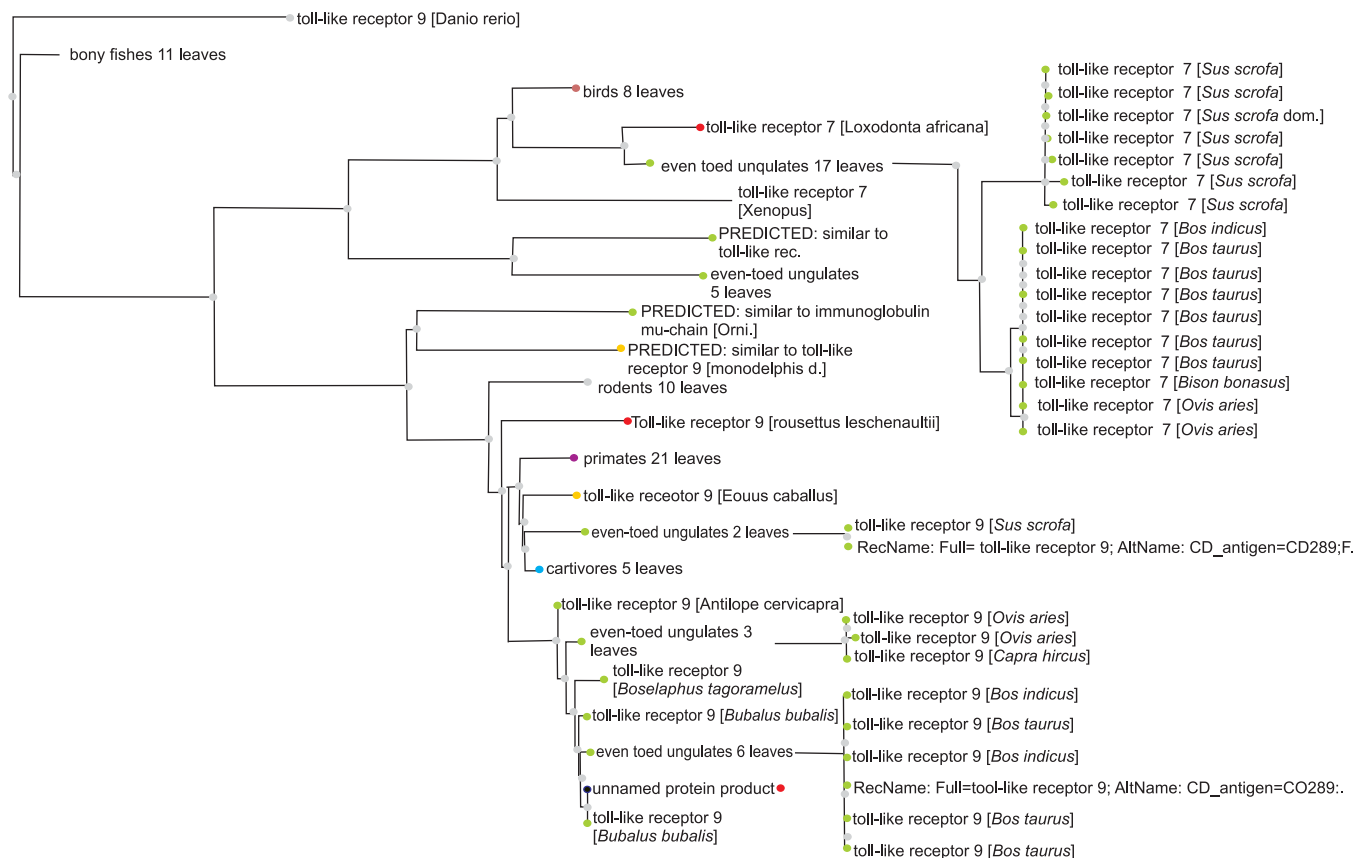


Fig. 4. Phylogenetic tree of TLR9 based on amino acid sequences.

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