Indian Journal of Animal Sciences **85** (3): 252–257, March 2015/Article

Cloning and molecular characterization of toll-like receptor 4 (TLR-4) gene in Indian water buffalo

RABIA BHARDWAJ[1], G S BRAH[2], J S ARORA[3], SIMARJEET KAUR[4] and C S MUKHOPADHYAY[5]

Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141 004 India

Received: 20 January 2013; Accepted: 20 October 2014

ABSTRACT

TLR4 is one of the best characterized TLRs and is mainly activated by lipopolysaccharide (LPS), a component of gram-negative bacteria. It has been implicated in signal transduction events induced by LPS found in most gram-negative bacteria. The present study was carried out to clone, sequence and to *in silico* characterize TLR4 gene of Indian water buffalo (*Bubalus bubalis*). For this, primer-pair (viz. TLR4-2B, TLR4-3B and TLR4-5B) were designed from TLR4 gene complete cds sequences available on NCBI database. Peripheral blood sample was collected by direct puncturing of the jugular vein of apparently healthy Murrah buffalo and was processed to isolate peripheral blood mononuclear cells (PBMCs). Total RNA was extracted from PBMCs, cDNA was synthesized immediately and TLR4 cDNA was amplified. PCR amplification resulted in amplicons of 863bp, 871 bp and 899 bp, respectively. The amplified product was cloned in TA cloning vector. Recombinant clones were selected by blue-white screening on selective medium (LB agar containing X-gal, IPTG and ampicillin). Randomly 6 white colonies were picked and grown in LB broth containing ampicillin (100µg/ml). Plasmids were isolated from clones following standard protocol and presence of insert was confirmed by PCR as well as restriction digestion by *EcoRI*. Positive clones were subjected to custom sequencing and the data obtained were analyzed. The deduced sequence was submitted to NCBI. Phylogenetic analysis indicated that the gene is conserved through evolution. The study suggested that TLR4 cds is highly conserved among the distant species under animal kingdom.

Key words: *Bubalus bubalis*, Cloning, Evolution, Lipopolysachharide (LPS), TLR4

The TLR multi-gene family encodes important recognition receptors that form the primary component of the afferent arm of the innate immunity; these were named after homology to *Drosophilla* Toll. Comparative sequence analysis of TLR4 exons revealed per cent identity of 95.7% to 100% at nucleotide and amino acid level, respectively with *Bos indicus* and *Bos taurus* breeds from different parts of the world (Malik *et al.* 2011). Current knowledge of the TLRs indicates that these are germ line encoded; membrane bound pattern-recognizing receptors (PRRs) that are essential elements in host defense, against pathogens by activating the innate immunity, a prerequisite for induction of adaptive immunity (Kwai and Akira 2011). Toll-like receptors play a major role in pathogen recognition and initiation of inflammatory immune responses which are triggered by dendritic cells (DCs) and macrophages, thereby bridging the innate and adaptive immunity (Szabo and Rajnavolgyi 2013).The growing interest in TLRs would bring a more complete understanding of the role of TLR-mediated responses and increase our range of weapons to treat infectious and immune diseases.The present study was carried out to clone, sequence and to *in silico* characterize the TLR4 gene of Indian water buffalo (*Bubalus bubalis*).

Present address: [1]Ph.D. Scholar (bhardwajrabia@gmail.com), [2]Adjunct Professor (gsbrah@gmail.com), [3,5]Assistant Scientist (drarora2003@gmail.com, csmbiotech@gmail.com), School of Animal Biotechnology, [4]Assistant Animal Geneticist (simarsharma08@gmail.com), Department of Animal Genetics and Breeding.

MATERIALS AND METHODS

Collection of blood samples: Around 15 ml of peripheral blood was aseptically collected in 0.5M EDTA (as anticoagulant) using sterilized 16G needle, by direct puncturing of jugular vein of adult and apparently healthy Murrah buffaloes, maintained at Dairy Farm of the university. The blood sample was immediately processed for PBMC isolation and extraction of total RNA and for their culture.

PBMC isolation, total RNA extraction and cDNA synthesis: Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood by density gradient centrifugation, using Histopaque 1077 and total RNA was isolated from PBMCs using Trizol as per the recommended protocol. The RNA was given DNase treatment to remove all contaminating DNA. RNA concentration and purity was checked using the Nano drop. The ratio of absorbance i.e. 260/280 should be 1.9 to 2.0 for RNA sample; 1000ng/µl of total RNA was used for cDNA synthesis using First cDNA synthesis kit as per the prescribed protocol.

PCR amplification of TLR4 gene: A set of TLR4 cDNA specific primers was designed, from the TLR4 mRNA complete cds of *Bubalus bubalis*, (accession No.JN608793), using Primer3 software (http://frodo.wi.mit.edu). These primers were got custom synthesized by the Integrated DNA Technologies. The quality of the designed primers was checked by analyzer and Primer BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) online tools. The sequence of the primers designed and used are TLR4-2B F:5'-*gcatggagctgaatctctac*- 3'; TLR4-2B R 5'-*gaagggcttgtagacttcct*-3', TLR4-3B F 5'-*cccctactcaacctctctttt*-3'; TLR4-3B R 5'-*gaagatgtcagggagcaag*-3', TLR4-5B F 5'-*actccctctctagccttcag*-3'; TLR4-5B F 5'-*ggcttttctgagtcttctcc*-3'. Primers were dissolved in sterile triple distilled water to make the stock (100 pmol/µl) and working solution of primers was prepared from the stock to achieve a concentration of 20pmol/µl.

The PCR assay was optimized using 25 µl reaction mixture. Ingredients of the PCR master mix was 1X PCR buffer, 1.5–2.5 mM $MgCl_2$, 200 µM dNTP mixes, and 0.5µM of each of forward and reverse primers, 1 U of Taq DNA polymerase and approx. 100 ng of cDNA. Cycling conditions for PCR amplification included one cycle of initial denaturation at 94°C for 5 min followed by 35 cycles each of denaturation (94°C for 1 min), annealing (48°C for 1 min) and extension (72°C for 1 min) followed by final extension at 72°C for 10 min. The PCR product was analyzed by running on 1.5% agarose gel prepared in 0.5X TBE buffer and visualized on gel documentation system.

Cloning of TLR4 amplicons in TA cloning vector: PCR was repeated in bulk (100µl) under standardized conditions and amplified product was run on 0.8% agarose gel, prepared in 0.5X TBE buffer for gel purification of the PCR product. Gel extraction of the TLR4 amplicon was done using Gel extraction kit as per the protocol provided by the manufacturer. Gel purified PCR product was successfully ligated with linearized pGEMT Easy™ cloning vector following manufacturer's instructions and cloned into DH5α strain of *E. coli* as per the protocol given by Sambrook and Russell (2001). Competent *E. coli* (DH5α) cells were prepared by calcium chloride ($CaCl_2$) method and transformation of ligated product into freshly prepared DH5α competent cells was done. Transformed cells were mixed with SOC (super optimal broth with catabolite) repressor broth at 37°C under shaking, plating was done on LB agar plates containing ampicillin (100µg/ml), X- gal (20 µg/ml) and IPTG (50 µg/ml) with the help of sterile L-spreader. Randomly, 6 white colonies were picked and inoculated in LB broth containing ampicillin (100µg/ml) and kept overnight at 37°C under shaking at 200 rpm. Plasmids were isolated from these clones by using plasmid miniprep kit. Positive clones were confirmed by PCR and restriction digestion by using enzyme *EcoRI,* inserts of specific sizes were released and resolved in 1.5% agarose gel, and finally the clones were sent for sequencing (Fig. 1). Sequencing data obtained were analyzed using various bioinformatics software and submitted to NCBI.

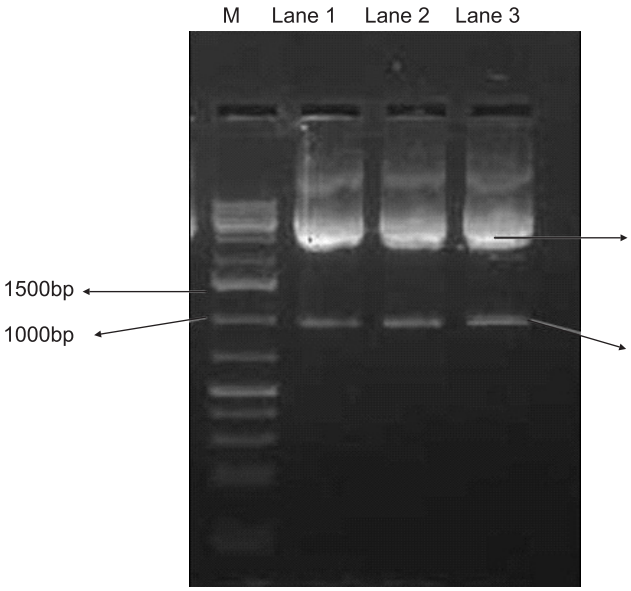

Fig. 1. RE digestion of plasmid from clone carrying insert Lane 1,2,3-*EcoRI*digested plasmid showing release of insert Marker- GeneRuler 1kb plus DNA ladder.

Nucleotide sequencing of the gene and phylogenetic analysis: Isolated plasmids were reconfirmed by PCR and restriction digestion and sent for commercial sequencing (using universal M13 forward and reverse primers) to DNA sequencing facility, Department of Biochemistry, Delhi University, New Delhi, India. The obtained nucleotide sequences (forward and reverse) of TLR4 gene sequence were displayed and analyzed using BioEdit sequence alignment editor, Version 7.0.9.0 (Hall 1999). Vector sequences at both 5' and 3'-termini of forward and reverse strands were trimmed. The open reading frame was deduced and the sequence was submitted to NCBI GenBank. The nucleotide sequence was subjected to BLASTn (Altschul *et al.* 1997) to compare for sequence similarities with other sequences available in the NCBI database (http://blast.ncbi.nlm.nih.gov/). The related sequences from other species were retrieved from GenBank and the equivalent sequences were selected and saved in FASTA format and aligned using alignment explorer/CLUSTAL of MEGA 5.0 software (Tamura *et al.* 2011). Nucleotide sequences of TLR4 gene of 41 divergent species from animal kingdom were subjected to multiple sequence alignment (MSA) using online software ClustalW2 (Larkin *et al.* 2007) with the following parameters gap open penalty: 10; end gap: false; gap extend penalty: 0.2; gap distance: 5. The cDNA sequences were used to construct the phylogenetic tree using maximum likelihood (MEGA 5.0 software) method with bootstrap re-sampling based on 1000 bootstrapped replications. The MEGA 5.0 software package was also used for:

- estimation of codon-based evolutionary divergence between sequences from the number of non-synonymous differences per non-synonymous site, number of synonymous differences per synonymous

site, and difference between the synonymous and non-synonymous changes per site from between sequences, using the modified Nei-Gojobori (assumed transition/transversion bias = 2) model (Zhang *et al.* 1998);

- estimation of disparity index, by calculating the homogeneity of substitution pattern between sequences (Kumar and Gadagkar 2001);
- Fisher's exact test of neutrality in favor of the alternative hypothesis of positive selection during evolution of TLR4 gene (Zhang *et al.* 1998), using the sequences included in the study.

RESULTS AND DISCUSSION

Total RNA extracted using standard protocol showed an $OD_{260/280}$ ~1.99 indicating the purity of the RNA preparation. PCR amplification of TLR4 gene resulted in 863 bp, 871 bp and 899 bp amplicons on 1.5% agarose gel (Fig. 2). Three sequences TLR4.2, 4.3 and 4.5 were joined together according to their overlapping regions. The gap from 1361bp to 1618 bp was bridged by using 'N' nucleotides.

BLAST results of the sequence: The BLASTn search result using the TLR4 cDNA sequence (of present study) coding sequence (cds) revealed a high degree of homology between the query sequence and other subject sequences available in the NCBI database, ranging between 86% with O*vis aries* (DQ922636) and 50% with *Danio rerio* (NM212813). Partial and predicted sequences vis-à-vis sequences showing degree of homology less than 50%, during BLASTn search, were discarded. Cloning and sequencing of complete TLR7 gene from local descript cattle (*Bos taurus)* of western Himalayan region revealed 98.9 to 99.6% amino acid sequence identity with different breeds of cattle and 87.1 – 98.9% identity with bubaline, equine, ovine, swine etc. Phylogenetic analysis revealed that nearest common ancestry is with *Bubaline* species (*Bubalus bubalis)* (Chakravarti *et al.* 2014). BLAST analysis for sequence alignment of TLR-4 gene of Murrah buffalo revealed 99, 98 and 80% homology with O*vis aries, Capra hircus* and *Homo sapiens* respectively (Mitra *et al.* 2011).

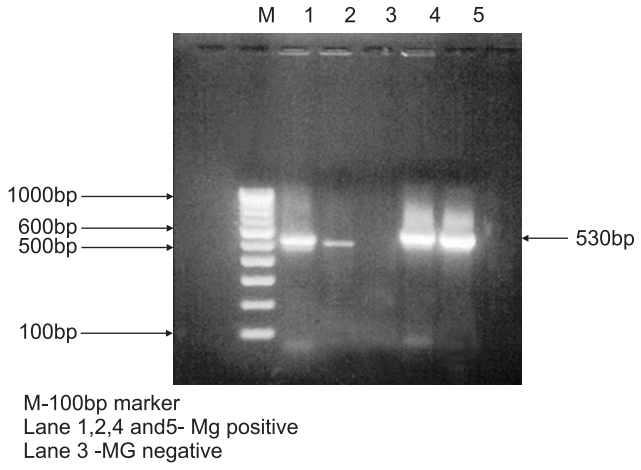

Fig. 2. PCR amplification of *TLR4-2-3*gene. Lane 1, PCR product of TLR4.5 gene; Lane 2. PCR product of TLR4. 3 gene; Lane 3, PCR product of TLR4.2 gene. Marker- GeneRuler^{TM} 1kb plus DNA ladder.

Multiple sequence alignment (MSA): The sequence alignment revealed that the TLR4 cDNA is conserved in part of the sequences for all the divergent animal species, however, the sequences were more similar among the animals belonging to the same family. Estimation of number of base differences per sequence, from bubaline TLR4 cds ranged between minimum in ruminants to maximum in the members of aquatic species. The phylogenetic tree showed similar results as revealed by the estimated numbers of base differences per sequence.

Phylogenetic analysis: The evolutionary history was inferred by using the maximum likelihood method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000

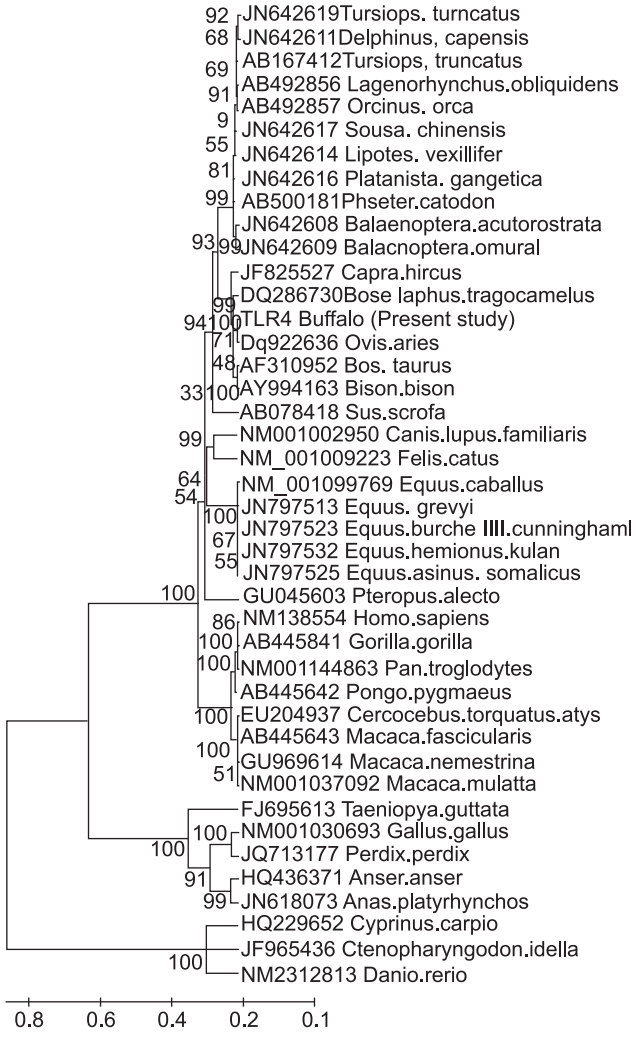

Fig. 3. Construction of molecular phylogenetic tree using maximum likelihood method with 1000 bootstrapped replications.

replicates) are shown next to the branches, bootstrap values greater than 90% were considered significant whereas the branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site, using MEGA5 (Tamura *et al.* 2011).

TLR4 cDNA (of present study) exhibited maximum divergence from the fresh water fishes *Cyprinus carpio* (HQ229652), *Ctenopharyngodon idella* (JF965436), *Danio rerio* (NM 212813), whereas dolphins and whales clustered very much near to the ruminants and were closely related to TLR4 cDNA (of present study) (Fig. 3). According to the phylogenetic tree, among the ruminants *Ovis aries* (DQ922636) was the closest, followed by *Bos taurus (*AF310952*)* and *Bison bison* (AY994163). Birds clustered separately, above the freshwater fishes, with significant bootstrap percentage (all greater than 90%). Members of the equine family were also clustered together having bootstrap values of 100%. High level of sequence similarity in ruminants clearly reflected the association of these genes with innate immune responses. The variation observed a few places in gene sequences are attributed to differences in resistance/susceptibility to certain bacterial infections while acting as valuable markers for analysis study (Tantia *et al.* 2012). Full length transcripts of Toll-like receptor genes (1–10) of river buffalo revealed high bootstrap values after phylogenetic analysis and amino acid identity of bubaline TLRs with ruminants ranged between 86% to 100% and 41% to 91% with other vetebrates (Dubey *et al.* 2013).

Effect of selection on TLR4 coding sequences

Synonymous substitutions per synonymous site: The TLR4 cDNA of *Danio rerio* (NM212813) showed maximum values for synonymous mutations per synonymous site (Table1). The difference was the least (0.000±0.000) for the sequence of *Ovis aries* (DQ922636) with respect to bubaline TLR4 cds.

Among the other pairs of sequences, *Equus grevyi* (JN797513), *Equus asinus somalicus* (JN797525), *Equus burchellii cunninghami* (JN797523) and *Equus hemionuskulan* (JN797532) showed maximum values for synonymous mutations per synonymous site with *Danio rerio* (NM212813) i.e. 0.294±0.210. Minimum values for synonymous mutations per synonymous site (0.000±0.000) were obtained when sequences of *Equus burchellii cunninghami* (JN797523) was compared with *Equus asinus somalicus* (JN797525) and with *Equus grevyi* (JN797513). Also, the species *Equus hemionuskulan* (JN797532) revealed minimum values (0.000±0.000) for synonymous mutations per synonymous site with *Equus asinus somalicus* (JN797525), *Equus grevyi* (JN797513) and *Equus burchellii cunninghami* (JN797523). Other species having minimum values (0.000±0.000) for synonymous mutations per synonymous site were *Macaca fascicularis* (AB445643) with respect to *Macaca mulatta* (NM001037092), *Macaca nemestrina* (GU969614) with both *Macaca mulatta* (NM001037092) and *Macaca fascicularis* (AB445643).

Non-synonymous substitutions per non-synonymous site: Ctenopharyngodon idella (JF965436) revealed maximum value for non-synonymous mutations per non-synonymous site, whereas, members of ruminant family revealed minimum value with respect to bubaline TLR4 cds (Table1).

Among the other pair of sequences, *Taenio pyaguttata* (FJ695613) showed maximum value of 0.579±0.018 for non-synonymous mutations per non-synonymous site with *Danio rerio* (NM212813) and other species pair having minimum values (0.000±0.017) for non-synonymous mutations per non-synonymous site were *Equus hemionuskulan* (JN797532) with *Equus burchellii cunninghami* (JN797523).

Codon-based evolutionary divergence between sequences: Maximum value difference between the synonymous and non-synonymous changes per site from between sequences with respect to TLR4 cds in buffalo (Table 1) was seen in *Cyprinus carpio* (HQ229652). While minimum values for difference between the synonymous and non-synonymous changes per site from between sequences was seen in the members of ruminant family by *Ovis aries* (DQ922636),when compared with the TLR4 cds obtained from the present study.

Among other pair of sequences *Cyprinus carpio* (HQ229652) revealed maximum value for difference between synonymous and non-synonymous changes per site from between sequences with respect to *Anser anser* (HQ436371) (–0.330±0.025) and the species having minimum values (0.000±0.000) for difference between the synonymous and non-synonymous changes per site from between sequences were *Physeter catodon* (AB500181) with *Tursiops truncates* (AB167412) and *Equus hemionuskulan* (JN797532) with *Equus burchellii cunninghami* (JN797523).

The test statistic values for codon-based test of neutrality along with the respective level of significance are given in Table 1.The rates of synonymous and non-synonymous substitutions explain the evolutionary dynamics of molecular sequences and the relative influence of positive selection and neutral evolution (Gonzales *et al*. 2002).The non-synonymous mutations suggested strong selective pressure, as opposed by synonymous mutations which are largely immune to natural selection (Yang and Nielsen 2000).

Disparity index: Significant values for disparity index (P> 0.05) was seen in *Bos taurus (*AF310952*), Ovis aries* (DQ922636), *Bison bison* (AY994163), *Boselaphus tragocamelus* (DQ286730), *Sus scrofa* (AB078418), *Lagenorhynchus obliquidens* (AB492856), *Perdix perdix* (JQ713177), *Gallus gallus* (NM001030693), *Felis catus* (NM001009223), *Equus caballus* (NM001099769), *Equus hemionuskulan* (JN797532), *Equus burchellii cunninghami* (JN797523), *Equus grevyi* (JN797513), *Equus asinus somalicus* (JN797525) (Table 1).

The aforementioned observations are also supported by the phylogenetic analysis. The probability (P) of rejecting

Table 1. The number of synonymous and non-synonymous substitutions per synonymous (dS) and non-synonymous (dN) sites, respectively, and the difference (dS-dN) vis-à-vis estimation of the number of base differences per sequence and estimation of disparity index between bubaline TLR4 cds and that of from other species

Species	dN±SE	dS±SE	(dS-dN)±SE	No. of base diff/seq[1]	Estimates of disparity index/site
*Bos taurus (*AF310952*)*	0.026±0.005	0.016±0.006	–0.011±0.008	41.000±6.105	1.000*
Capra hircus (JF825527)	0.039±0.006	0.018±0.007	–0.021±0009	76.000±8.295	0.041
Ovis aries (DQ922636)	0.001±0.001	0.000±0000	–0.001±0.001	7.000±2.543	0.331*
Boselaphustrago camelus (DQ286730)	0.041±0.006	0.018±0.007	–0.023±0.009	59.000±7.020	1.000*
Bison bison (AY994163)	0.026±0.005	0.013±0.006	–0.013±0.007	41.000±6.232	1.000*
Orcinus orca (AB492857)	0.108±0.010	0.050±0.011	–0.058±0.014	176.000±10.704	0.075
Tursiops truncates (AB167412)	0.110±0.010	0.052±0.011	–0.058±0.014	183.000±11.004	0.195
Lagenorhynchus obliquidens (AB492856)	0.115±0.011	0.055±0.011	–0.060±0.015	186.000±11.145	0.300*
Balaenoptera acutorostrata (JN642608)	0.103±0.010	0.047±0.011	–0.056±0.014	173.000±11.662	0.132
Physeter catodon (AB500181)	0.111±0.011	0.050±0.011	–0.061±0.014	181.000±11.076	0.092
Sousa chinensis (JN642617)	0.107±0.011	0.047±0.011	–0.060±0.015	171.000±10.659	0.077
Platanista gangetica (JN642616)	0.107±0.010	0.052±0.012	–0.054±0.015	176.000±11.007	0.074
Lipotes vexillifer (JN642614)	0.110±0.010	0.050±0.011	–0.060±0.014	178.000±11.448	0.028
Balaenoptera omurai (JN642609)	0.106±0.010	0.052±0.011	–0.053±0.015	175.000±11.445	0.111
Sus scrofa (AB078418)	0.147±0.012	0.069±0.013	–0.078±0.016	250.000±13.481	1.000*
*Delphinus capensis (*JN642611)	0.113±0.011	0.047±0.011	–0.066±0.014	181.000±10.701	0.093
Pongo pygmaeus (AB445642)	0.196±.013	0.081±0.014	–0.114±0.019	335.000±16.051	0.009
Pteropus alecto(GU045603)	0.180±0.013	0.082±0.014	–0.098±0.018	299.000±15.795	0.190
Canis lupus familiaris (NM001002950)	0.186±0.012	0.089±0.014	–0.096±0.018	312.000±15.279	0.097
Macaca nemestrina (GU969614)	0.192±0.013	0.076±0.013	–0.116±0.019	330.000±16.204	0.000
Macaca fascicularis (AB445643)	0.194±0.013	0.076±0.013	–0.118±0.019	332.000±16.077	0.002
Macaca mulatta (NM001037092)	0.195±0.013	0.076±0.013	–0.119±0.019	331.000±16.123	0.002
Cercocebustorquatus atys (EU204937)	0.197±0.013	0.080±0.013	–0.118±0.018	337.000±16.409	0.010
Gallus gallus (NM001030693)	0.461±0.016	0.220±0.019	–0.242±0.024	803.000±20.301	1.000*
Perdix perdix (JQ713177)	0.466±0.017	0.211±0.019	–0.255±0.024	805.000±20.001	1.000*
Anser anser (HQ436371)	0.471±0.016	0.213±0.020	–0.258±0.025	806.000±19.849	0.234
Anas platyrhynchos (JN618073)	0.480±0.016	0.212±0.020	–0.268±0.025	814.000±19.811	0.178
Taenio pyaguttata (FJ695613)	0.493±0.017	0.229±0.020	–0.264±0.025	822.000±19.900	0.000
Homo sapiens (NM138554)	0.195±0.013	0.084±0.014	–0.111±0.019	334.000±15.696	0.008
Pan troglodytes (NM001144863)	0.194±0.013	0.087±0.014	–0.108±0.019	335.000±15.827	0.013
Gorilla gorilla (AB445641)	0.197±0.013	0.084±0.013	–0.113±0.019	335.000±15.791	0.015
Felis catus (NM001009223)	0.178±0.012	0.078±0.013	–0.100±0.017	303.000±14.614	0.341*
Cyprinus carpio (HQ229652)	0.570±0.017	0.263±0020	–0.307±0.025	918.000±20.560	0.000
Ctenopharyngodon idella (JF965436)	0.571±0.016	0.269±0021	–0.301±0.024	912.000±19.886	0.000
Danio rerio (NM212813)	0.562±0.016	0.277±0021	–0.285±0.025	916.000±21.146	0.000
Equus caballus (NM001099769)	0.170±0.012	0.084±0.014	–0.086±0.018	290.000±15.440	1.000*
Equus hemionuskulan (JN797532)	0.170±0.012	0.086±0.014	–0.084±0.018	291.000±15.382	1.000*
Equus burchellii cunninghami (JN797523)	0.170±0.012	0.086±0.014	–0.084±0.018	291.000±15.382	1.000*
Equus asinus somalicus (JN797525)	0.171±0.012	0.086±0.014	–0.085±0.018	293.000±15.481	1.000*
Equus grevyi (JN797513)	0.171±0.012	0.086±0.014	–0.085±0.018	292.000±15.401	1.000*

* P<0.05.1 estimated number of base differences per sequence.

the null hypothesis of strict-neutrality in favor of the alternative hypothesis of positive selection was significant for all the sequences, except for *Bos taurus* (AF310952), *Ovis aries* (DQ922636) and *Bison bison* (AY994163). This suggested that the sequences have experienced positive selection during the process of evolution.

The synonymous and non-synonymous substitutions per the respective sites indicate that substitutions were the highest for the aquatic species followed by the avians. Our result supported the hypothesis that TLR4 has evolved separately from the animal species belonging to aquatic and avian origin. The null hypothesis of strict-neutrality in favor of the alternative hypothesis of positive selection, between bubaline TLR cds and other sequences has been rejected. Hence, purifying selection has played major role in evolution of the TLR4 sequences. The present study clearly indicated that there exists less genetic variability for TLR4 sequence among divergent species, which signifies that TLR4 gene is highly conserved among different species.

The overall study clearly indicated that the sequences experienced purifying selection (among the ruminant species) during evolution. The TLR4 gene is required to maintain fitness of an individual by defending against invasion of Gram-negative organisms.

ACKNOWLEDGEMENT

The authors thankfully acknowledge the funds provided under the Rashtriya Krishi Vigyan Yojna (National Agricultural Science Scheme), Government of India, for the research work.

REFERENCES

Altschul S F, Madden T L, Schaffer A A, Zhang J, Zhang Z, Miller W and Lipman D J. 1997. Gapped BLAST and PSI BLAST: A new generation of protein database search programs. *Nucleic Acids Research* **25:** 3389–402.

Chakravarti S, Biswas S, Mohapatra J K, Sharma K, Vaid N, Rajak K K, Singh R K, Mondal B and Malik Y P S. 2014. Phylogenetic and homology analysis of toll-like receptor gene TLR7 in *Bos indicus* from high altitude areas of Uttarakhand state, India. *Agricultural Research* **3**(1): 92–96.

Dubey P K, Goyal S, Kathiravan P, Mishra B P, Gahlawat S K and Kataria R S. 2013. Sequence characterization of river buffalo Toll-like receptor genes 1–10 reveals distinct relationship with cattle and sheep. *International Journal of Immunogenetics* **40**(2): 140–48.

Gonzales M, Dugan J and Shafer R. 2002. Synonymous-non-synonymous mutation rates between sequences containing ambiguous nucleotides (Syn-SCAN). *Bioinformatics* **18**: 886–87.

Hall T A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis. 95–98.

Kawai T and Akira S. 2011.Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* **34**(5): 637–50.

Kumar S and Gadagkar S R. 2001. Disparity Index: A simple statistic to measure and test the homogeneity of substitution patterns between molecular sequences. *Genetics* **158**: 1321–27.

Larkin M A, Blackshields G, Brown N P, Chenna R, McGettigan P A, McWilliam H, Valentin F, Wallace I M, Wilm A, Lopez R, Thompson J D, Gibson T J and Higgins D G. 2007. Clustal W and clustal X version 2. *Bioinformatics* **23**(21)**:** 2947–48.

Malik Y P S, Chakravarti S, Sharma K, Vaid N, Rajak K K and Singh R K. 2011. Genomic analysis of Toll-like Receptor 4 and 7 exons of *Bos indicus* from temperate and sub-Himalayan region of India. *Journal of Animal Sciences* **7:** 1019–25.

Mitra M, Taraphder S, Sonawane G S and Verma A. 2012. Nucleotide sequencing and SNP detection of toll-like receptor-4 gene in Murrah buffalo (*Bubalus bubalis*). *International Scholarly Research Network* **2012**: (dx.doi.org 10.5402/2012/659513).

Sambrook J and Russell D W. 2001. *Molecular Cloning: A Laboratory Manual*. 3rdedn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Szabo A and Rajnavolgi E. 2013. Collaboration of Toll-like and RIG-I-like receptors in human dendritic cells: tRIGgering antiviral innate immune responses. *American Journal of Clinical and Experimental Immunology* **2**(3): 195–207.

Tantia M S, Mishra B, Banerjee P, Joshi J, Upasna S and Vijh R K. 2012. Phylogenetic and sequence analysis of toll like receptor genes (TLR-2 and TLR-4) in buffaloes. *Indian Journal of Animal Sciences* **82**(8): 875–78.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**(10): 2731–39. doi: 10.1093/molbev/msr121

Yang Z and Nielsen R. 2000. Estimating synonymous and nonsynonymous substitution rates under realistic evolutionary models. *Molecular Biology and Evolution* **17:** 32–43.

Zhang J, Rosenberg H F and Nei M. 1998. Positive Darwinian selection after gene duplication in primate ribonuclease genes. *Proceedings of the National Academy of Sciences (USA)* **95:** 3708–13.