Comparative Analysis of Molecular Structure, Function and Expression of Buffalo (*Bubalus bubalis*) Toll-Like Receptor 9

Anju Manuja[1,*], Balvinder K. Manuja[2], Ranjit S. Kataria[3], Ramesh K. Sethi[1] and Raj K. Singh[2]

[1]*Central Institute for Research on Buffaloes, Sirsa Road, Hisar-125001, Haryana, India*

[2]*National Research Centre on Equines, Sirsa Road, Hisar-125001, Haryana, India*

[3]*National Bureau of Animal Genetic Resources, GT Road Bye Pass, Karnal, India*

Abstract: Toll-like receptor 9 (TLR9) has been characterized as a receptor that recognizes unmethylated CpG motif and triggers a pro-inflammatory cytokine response that influences both innate and adaptive immunity. Buffalo is an economically important livestock species in many Asian and Mediterranean countries, but there is little information available on its TLR9 structure and response to stimulation with its agonist CpG-ODNs. Hence in this study, we report the analysis of newly sequenced buffalo TLR9 gene fragment. In this study, buffalo TLR9 amino acid sequence revealed close association of TLR9 proteins within other bovines and small ruminants; but high divergence from other species. Multiple alignment of deduced amino acid sequence of *Bubalus bubalis* TLR9 with other species showed that 156/201 (74.28%) amino acids were conserved in all species. Leucine rich repeat (LRR) motifs in the ectodomain of TLR9 are responsible for molecular recognition of its agonist. The LRR pattern of *Bubalus bubalis* TLR9 protein was predicted towards N-terminal sequence and was found to be conserved among all species except *Rattus norvegicus* and *Equus caballus.* Blast analysis of buffalo TLR9 sequence with single nucleotide polymorphisms (SNPs) database revealed 13 SNPs out of which 7 were cds-synonymous and 6 were of the functional significance. Furthermore, kinetics of TLR9 and proinflammatory IL-1β and TNF-α cytokine expression by buffalo PBMCs influenced by CpG-ODN is also discussed.

Keywords: *Bubalus bubalis*, TLR9, CpG-ODN, LRR, IL-1, TNF-α, Single nucleotide polymorphism.

1. INTRODUCTION

Toll-like receptors (TLRs) constitute an ancient superfamily of proteins that are expressed in both vertebrate and invertebrate species. In mammals, 13 TLRs (TLRs 1-13) have been identified although expression of TLRs 10-13 appears to be species specific [1]. TLR21 has been identified in fish and amphibians and TLR15 in chickens only [2, 3]. The ligands for these receptors are components of pathogenic microbes and are often called as Pathogen Associated Molecular Patterns (PAMPs). Non-methylated CpG motifs present in viral and bacterial DNA are one of the PAMPs recognized by mammalian innate immune system. TLR9 has been characterized as a receptor that can recognize unmethylated CpG motif and trigger a pro-inflammatory cytokine response that influences both innate and adaptive immunity.

Synthetic CpG-oligodeoxynucleotides (CpG-ODN) mimic bacterial DNA and shown to have potent immunostimulatory activity in vertebrates [4]. *In vivo*, CpG-ODNs have been shown to induce strong type 1 immune responses, with subsequent activation of cellular (cytotoxic T lymphocytes, CTLs) and humoral (Th1 immunoglobulin isotypes) components [5]. Therefore, CpG-ODNs have been extensively studied for their application as adjuvants in vaccines in domestic animals, including bovines, ovines and swines [6]. It has been observed that subtle differences exist in different species in response to CpG-ODN. Significant structural differences in the extracellular domain of TLR9 account for species-specific recognition of CpG ODN sequences [7].

Buffalo (*Bubalus bubalis*) is an economically important livestock species in many Asian and Mediterranean countries, but there is no information available on its TLR9 structure and response to its stimulation with CpG-ODNs. N-terminal site in TLR9 is responsible for CpG-DNA recognition and activation [8]. Hence in this study, we report the molecular analysis of TLR9 gene fragment for the first time and quantitative expression of TLR9 and pro-inflammatory cytokines by peripheral blood mononuclear cells (PBMCs) of buffalo calves in response to stimulation with CpG-ODNs.

2. MATERIAL AND METHODS

2.1. Isolation of PBMCs and RNA Extraction

Blood samples were collected from buffalo calves and PBMCs were separated by density gradient centrifugation on histopaque (Sigma, St Louis) as per

*Address correspondence to this author at the National Research Centre on Equines, Hisar-125001, Haryana, India; Tel: 919466127780;
Fax: 91 1662 276217; E-mail: amanuja@rediffmail.com

ISSN: 1927-5196 / E-ISSN: 1927-520X/13

manufacturer's instructions. The cells were washed thrice with PBS by centrifuging at 200 × *g* for 10 min at room temperature. The viable cells were counted by trypan blue dye exclusion technique, resuspended at 10^7 cells per ml in PBS and used for RNA extraction.

Total RNA was extracted from 1 × 10^7 PBMCs using RNeasy kit (Qiagen GmBH, Hilden, Germany), as per manufacturer's instructions and treated with DNase I (Fermentas, Lithuania) onto the column in order to remove genomic DNA. The quality and quantity of RNA was determined by spectrophotometer (Picodrop, Saffron Walden) by taking the absorbance at 260 nm and 280 nm. The RNA samples having A_{260}/A_{280} ratios from 1.9 to 2.1 were used in further experiments.

2.2. Amplification, Cloning and Sequencing of TLR9 Transcripts

Total RNA was used for first strand synthesis using oligo dT with MMLV reverse transcriptase enzyme. Consensus primers were designed to amplify partial TLR9 sequence towards N-terminal by aligning reported TLR9 mRNA sequences (Table **1**). TLR9 gene towards N-terminal was amplified by polymerase chain reaction (PCR) with thermal conditions of 5 min at 94°C and 30 cycles of denaturation at 94°C for 30 sec, annealing 55°C for 30 sec, extension at 72°C for 30 sec and finally extension at 72°C for 5 min. The PCR products were checked by agarose gel (1%) electrophoresis. Amplicons were cloned into pUC18 vector. The recombinant positive clones were selected by blue white screening. The white colonies were picked and incubated in Luria Britanni broth. Confirmation of clones was done by restriction digestion with Hind III and agarose gel (1%) electrophoresis.

2.3. Analysis of Amino Acid Sequences and Predicted Proteins

Nucleotide sequence data obtained was analysed using blastn. The amino acid sequences deduced from the nucleotide sequence and sequences of the TLR9 of different species retrieved from GenBank were aligned by ClustalX. Phylogenetic tree based on the deduced amino acid alignments was constructed by neighbor-joining method using MEGA 4 [9]. The percent sequence distances were calculated for TLR9 amino acid sequences of related species. Amino acid substitutions were analysed by multiple alignments in ClustalX and graphically presented using Bioedit program. The online utility SMART (http://smart.embl-heidelberg.de/) was used for comparative prediction of protein domain architectures. Specialised blast of buffalo TLR9 sequence to search single nucleotide polymorphisms within available SNPs database was performed online (www.ncbi.nlm.nih.gov/SNP/snp_blastByOrg.cgi).

2.4. Cell Culture and Stimulation

PBMCs from three buffalo calves were cultured in RPMI-1640 (Sigma-Aldrich, St Louis) supplemented

Table 1: Primers for Conventional and Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Gene	Primer/Probe	Sequence 5'-3'	Amplicon length (bp)
TLR9	Forward	CCT GCC CTG TGA GCT CCA	605 bp
	Reverse	TGACAATGTGGTTGTAGGACA	
TLR9	Forward	CAACAGCATCTCGCAGGC GGTTAAT	100 bp
	Reverse	CATGGTACAGGTCCAGCTTGT TGTG	
	Probe	CCAGTTCGTGCC GCTGACCAGCCTGCG AGT	
IL-1β	Forward	ACCTTCATTGCCCAGGTTTCT	120 bp
	Reverse	CTGTTTAGGGTCATCAGCCTCAA	
	Probe	ACAGCTCATTCTCGTCACTGTAGTAAGCCATCA	
TNF-α	Forward	CCACGTTGTAGCCGACATCA	100 bp
	Reverse	CTGGTTGTCTTCCAGCTTCACA	
	Probe	ACTCGTATGCCAATGCCCTCATGGC	
GAPDH	Forward	TTCTGGCAAAGTGGACATCGT	112 bp
	Reverse	CTTGACTGTGCCGTTGAACTTG	
	Probe	ACATGGTCTACATGTTCCAGTATGATTCCACCC	

with 10% fetal bovine serum (Sigma Aldrich), 50 µg/ml streptomycin sulfate, 10 µg/ml gentamicin sulfate, 2 mM L-glutamine (Sigma-Aldrich), 50 µM 2-mercaptoethanol. Cells were incubated in six well round bottom plates (NUNC) using culture medium at 37°C in an atmosphere of 5% CO_2 and 95% humidity. After 24 hours, the cells were stimulated with class A CpG-ODN 2216 and GpC control (InvivoGen, San Diego, California USA) at 2 µg/ml for 6, 12, 24 and 48 h.

2.5. RNA Extraction and Reverse Transcription

Total RNA was extracted from PBMCs stimulated with CpG-ODN, GpC control and unstimulated PBMCs. Reverse transcription was performed by Enhanced Avian RT First Strand Synthesis kit (Sigma-Aldrich) in a 20 µl final volume containing 1 µg of total RNA, 500 µM dNTPs, 3.5 µM Oligo $(dT)_{23}$ incubated at 70°C for 10 min to denature RNA secondary structure. Purified avian myeloblastosis virus reverse transcriptase (eAMV-RT), RNase inhibitor both @ 1 unit/µl along with the buffer containing 50 mM Tris-HCl, pH 8.3, 40 mM KCl, 8 mM $MgCl_2$, 1 mM DTT was added and incubated at 42°C for 50 min and stored at –20°C for further use.

2.6. Relative Quantitation of TLR9, Cytokine IL-1β and TNF-α

Quantitative PCR for TLR9, IL-1β, TNF-α and GAPDH (house keeping gene) was performed in 25 µl reactions with JumpStart *Taq* Ready Mix (Sigma-Aldrich, USA), 3 µl of cDNA, 600 nM concentrations of forward and reverse primers and 200 nM of probe. The sequences of primers and probes used for qRT-PCR as described earlier for IL-1β, TNF-α and GAPDH [10] and TLR9 are listed in Table **1**. Each sample was amplified in duplicates in the same 96-well PCR plate, sealed with optical sealing tape using a Miniopticon Real Time PCR detection system (BIO-RAD, Hurcules, California, USA). Thermal cycling conditions were 3 min at 95°C and 45 cycles of 15 sec at 95°C and 45 sec at 60°C. The real time PCR specificity was also confirmed with melting curve analysis. The threshold cycle numbers (C_t) were automatically determined by the Opticon Monitor^{Tm} version 3.1. Each sample was amplified for GAPDH and specific gene simultaneously in the same plate to avoid plate to plate variation.

TLR9, IL-1β and TNF-α mRNA expression was measured by relative quantification, by comparing the threshold cycle (Ct) of PBMCs stimulated with CpG and GpC to the Ct generated by a reference sample (PBMCs control) and calibrator (GAPDH). TLR9 gene expression was normalised to GAPDH and used to calculate the relative fold difference in gene expression in different samples using the equation $2^{-\Delta\Delta}$Ct [11].

3. RESULTS

3.1. TLR9 mRNA Expression and Cloning of TLR9 Gene

The primary PCR products generated by RT-PCR from total RNA extracted from PBMCs of buffalo calves using consensus primers yielded expected product of 605 bp of TLR9 gene. The amplified product of TLR9 gene was cloned into pUC18 vector. The positive clones were screened by blue-white colony selection and restriction digestion with Hind III. The plasmid DNA isolated from white colonies with inserts upon digestion

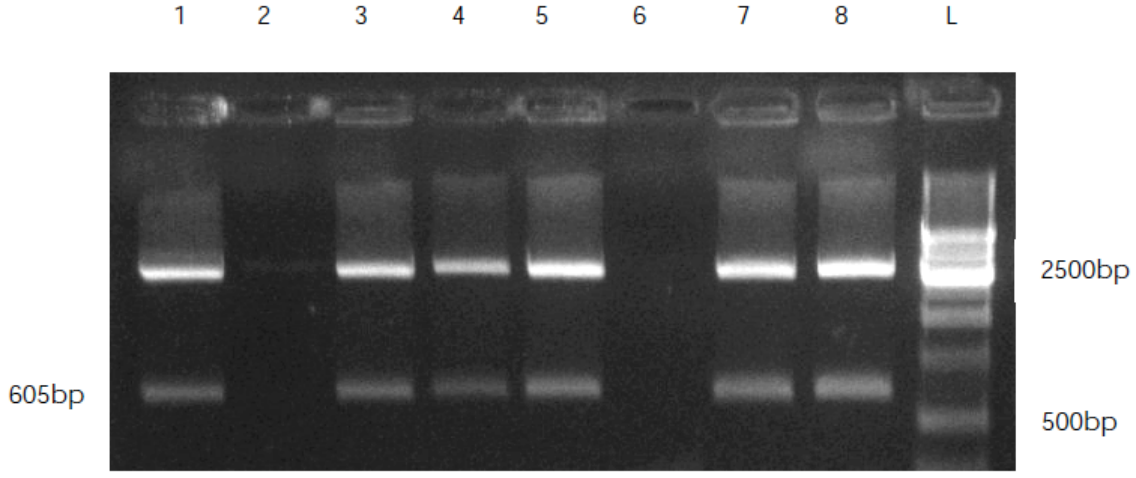

Figure 1: Restriction enzyme digested plasmids from positive recombinant clones. Lane description: 1-8: Plasmid DNA digested with Hind III enzyme. Positive clones: Lanes 1, 3, 4, 5, 7 and 8. L: 500 bp DNA ladder.

Table 2: Estimates of Evolutionary Divergence Between Sequences of TLR9 Gene. Percent Distances of Amino Acid Sequences of *Bubalus bubalis* (HQ283275) with TLR9 Gene of Different Species Retrieved from GenBank

Species	1	2	3	4	5	6	7	8	9	10	11	12
Bubalus bubalis HQ283275	0.00											
Mus musculus AB045181	0.22	0.00										
Homo sapiens DQ019997	0.17	0.22	0.00									
Gorilla gorilla AB445676	0.17	0.22	0.00	0.00								
Equus caballus NM_001081790	0.10	0.21	0.13	0.13	0.00							
Capra hircus EU747825	0.04	0.22	0.18	0.18	0.12	0.00						
Ovis aries NM_001011555	0.04	0.22	0.18	0.18	0.12	0.01	0.00					
Bos indicus EF076726	0.02	0.22	0.17	0.17	0.11	0.05	0.05	0.00				
Bos taurus EF076731	0.02	0.22	0.17	0.17	0.11	0.05	0.05	0.00	0.00			
Rattus norvegicus NM_198131	0.23	0.11	0.22	0.22	0.23	0.24	0.24	0.22	0.22	0.00		
Canis familiaris AY859723	0.15	0.21	0.14	0.14	0.14	0.14	0.14	0.17	0.17	0.24	0.00	
Felis catus AY859724	0.14	0.21	0.13	0.13	0.13	0.15	0.15	0.15	0.15	0.23	0.07	0.00

with Hind III restriction enzyme yielded two bands one for vector and another for 605 bp TLR9-specific PCR product (Figure **1**). The partial sequence of buffalo TLR9 obtained was submitted to the GenBank and has been assigned accession number HQ283275.

3.2. Nucleotide and Amino Acid Analysis

The amino acid sequences deduced from the nucleotide sequence of *Bubalus bubalis* in the present study (HQ283275) and sequences of the TLR9 of different species retrieved from GenBank (*Bubalus bubalis*, HQ283275; *Mus musculus,* AB045181; *Homo sapiens,* DQ019997; *Gorilla gorilla*, AB445676; *Equus caballus,* NM_001081790; *Capra hircus,* EU747825; *Ovis aries*,NM_001011555; *Bos indicus,* EF076726; *Bos taurus,* EF076731; *Bubalus bubalis,* FJ606787; *Ratus norvegicus,* NM_198131; *Canis familiaris,* AY859723; *Felis catus,* AY859724) were aligned by ClustalX and analysed. The deduced amino acid

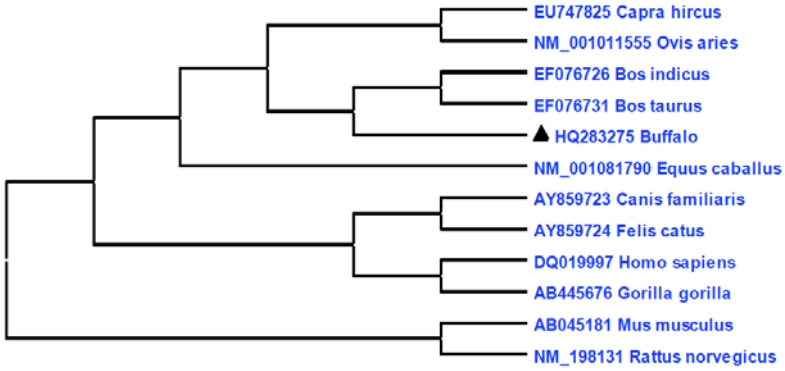

Figure 2: Phylogenic tree of deduced amino acid sequences of partial TLR9 mRNA of buffalo (*Bubalus bubalis,* HQ283275) indicating genetic relationship with TLR9 gene sequences of different species retrieved from GenBank database (*Mus musculus,* AB045181; *Homo sapiens,* DQ019997; *Gorilla gorilla*, AB445676; *Equus caballus,* NM_001081790; *Capra hircus,* EU747825; *Ovis aries*,NM_001011555; *Bos indicus,* EF076726; *Bos taurus,* EF076731; *Rattus norvegicus,* NM_198131; *Canis familiaris,* AY859723; *Felis catus,* AY859724). Distances and groupings were determined using the MEGA 4 software by neighbor joining method. The veracity of these trees was studied using the bootstrapping method by executing 500 replicates.

sequence was highly similar to TLR9 sequences of buffalo and cattle retrieved from GenBank (EF076726, EF076731, FJ606787). The estimates of evolutionary divergence between sequences of buffalo TLR9 gene with TLR9 genes of other species retrieved from GenBank revealed that the percent distances ranged from 0-23% (Table **2**). The divergence was 4% when compared to small ruminants like sheep and goats. However, the buffalo sequences were highly dissimilar as compared to mouse (22%), human (17%), gorilla (17%), rat (23%), dog (15%), cat (14%) and horse (10%).

On phylogenic analysis based on amino acid sequences of buffalo TLR9 in the present study (HQ283275) and sequences of other species retrieved from GenBank, TLR9 proteins from the present study clustered with bovine protein sequences (FJ606787),

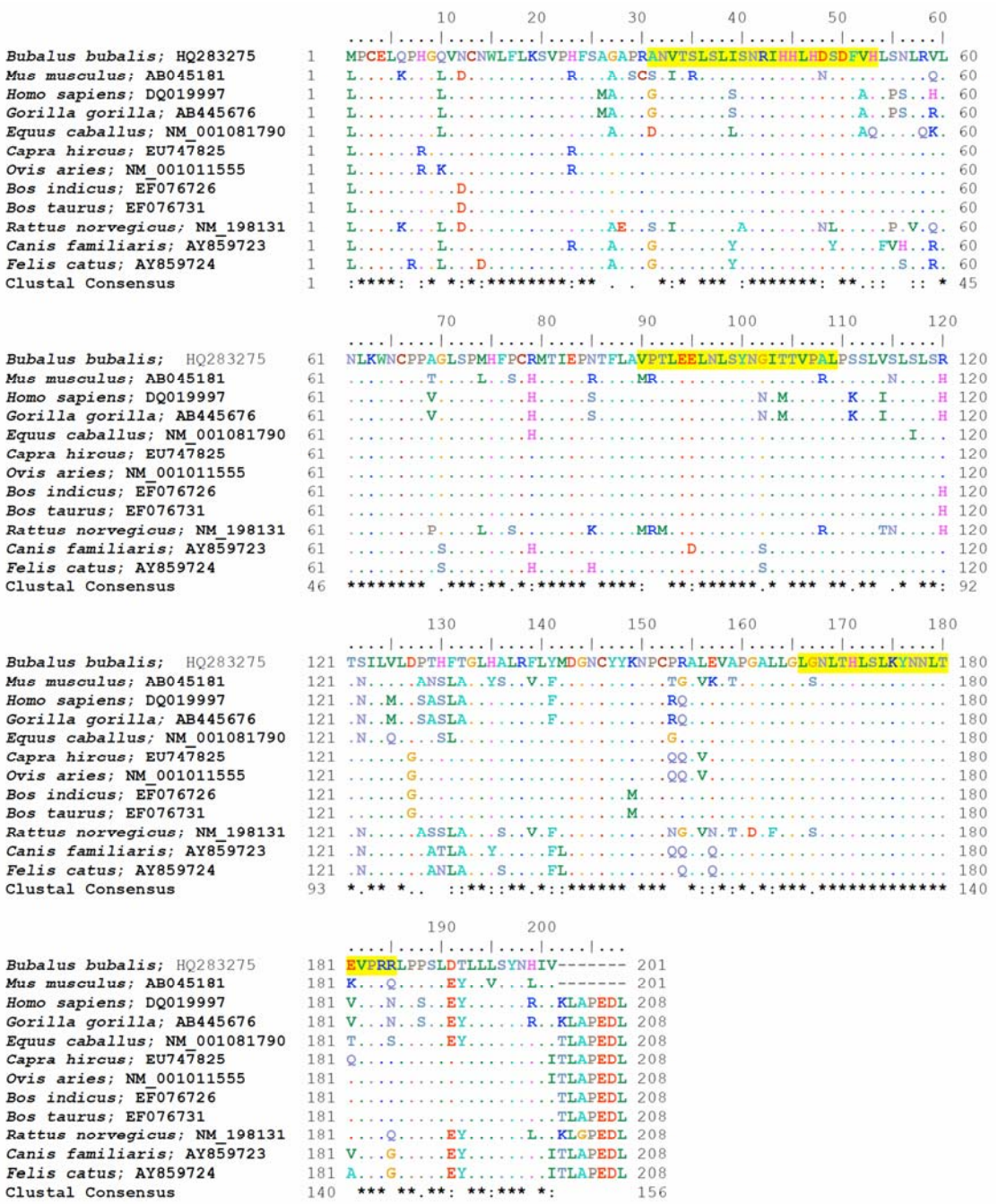

Figure 3: Comparison of deduced amino acid sequence of TLR9 of *Bubalus bubalis* (HQ283275) with other species retrieved from GenBank (*Mus musculus, Homosapiens, Gorilla gorilla, Equus caballus, Capra hircus, Ovis aries, Bos indicus, Bos taurus, Rattus norvegicus, Canis familiari,* and *Felis catus*) showing substitution of amino acids. The amino acids coding LRR regions in *Bubalus bubalis* are highlighted in yellow.

while human & gorilla, cattle (*Bos indicus* & *Bos taurus*), dogs and cats, sheep and goats, mice and rats and horse formed separate clades (Figure **2**). The analysis shows conserved sequences and close association of TLR9 proteins within related species of small and large ruminants whereas high divergence with other species. Multiple alignment of deduced amino acid sequence of TLR9 of *Bubalus bubalis* with other species revealed that 74. 28% (156/201) amino acids were conserved in all species. The substitution of amino acids is presented in Figure **3**.

Comparative prediction of TLR9 protein domain architectures for *Mus musculus, Homosapiens, Gorilla gorilla, Capra hircus, Ovis aries, Bos indicus, Bos taurus, Bubalus bubalis, Canis familiaris, and Felis catus* confidently predicted three leucine rich repeats (LRRs) in partial TLR9 sequence. LRR patterns were conserved among all species except *Equus caballus* and *Rattus norvegicus*.

Blast analysis of buffalo TLR9 sequence with single nucleotide polymorphisms (SNPs) database (http://www.ncbi.nlm.nih.gov/SNP/snp_blastByOrg.cgi), a total of 13 dsSNPs IDs (1 cow, 11 human, 1 mouse), with all possible genotypic classes (+/+; +/−; −/−) were observed. Overall, 76.97% (*n* = 10) of the SNPs observed were transitions (A ↔ G; C ↔ T) and 23.07% (n=3) were transversions. Of the 13 SNPs identified, 7 were cds-synonymous and 6 were of the functional significance. The allele change, corresponding mRNA and amino acid positions, and relevant dbSNPs accession numbers are described in Table **3**.

3.3. Expression of TLR9 and Cytokines IL-1β and TNF-α in Stimulated and Non-Stimulated Cells

Quantitative PCR was used to determine mRNA expression of TLR9 and GAPDH, in unstimulated PBMCs, PBMCs stimulated with CpG ODN 2216 and GpC ODN. The values for threshold cycles decreased with the increasing time starting from 6 to 48 h indicating higher expression of TLR9 in CpG stimulated PBMCs at 48 h indicating higher abundance of the TLR9. Relative quantitation with ΔC_t values for GpC and CpG stimulated PBMCs with reference to the C_t generated by unstimulated PBMCs are shown in Table **4**. PBMCs stimulated with CpG ODN exhibited 6-fold higher abundance of TLR9 at 24 h and 58-fold at 48 h of stimulation as compared to unstimulated PBMCs, whereas inverted sequences exhibited 5 fold higher expressions at 24 h and but decreased at 48 h (Figure **4A**).

IL-1β and TNF-α mRNA expressions were measured by qRT-PCR in PBMCs following stimulation with CpG ODN and GpC ODN. CpG stimulated cells exhibited 1323 fold higher IL-1β expression with respect to unstimulated PBMCs whereas GpC stimulated cells exhibited 1136 fold higher expression at 48 h of stimulation (Figure **4B**). CpG stimulated cells exhibited 1260 fold higher expression of TNF-α and

Table 3: Single Nucleotide Polymorphisms (SNPs) by Blast Analysis of *Bubalus bubalis* TLR9 with TLR9 of Different Species

Allele change	Species	dbSNP ID	mRNA/protein position	Function	Residue change*
C/T	Cow	rs55617136	422/100	Missense	**A to V**
A/G	Human	rs72959313	1138/168	Cds-synon	A to A
G/T	Human	rs116310431	1075/147	Cds-synon	S to S
C/T	Human	rs5743842	647/5	Missense	**R to C**
A/G	Human	rs352139	1075/147	Cds-synon	S to S
G/T	Human	rs55979550	807/58	Missense	**M to R**
A/G	Human	rs56287816	1001/123	Missense	**P to S**
C/T	Human	rs5743844	930/99	Missense	**P to L**
G/T	Human	rs5743843	871/79	Missense	**H to Q**
C/T	Human	rs56116373	976/114	Cds-synon	I to I
A/G	Human	rs55993803	778/48	Cds-synon	L to L
C/T	Human	rs55700905	889/85	Cds-synon	H to H
C/T	Mouse	rs29741158	349/81	Cds-synon	S to S

*predicted amino acid(s) encoded by alleles with predicted amino acid replacements depicted in bold.

Table 4: Relative Quantitation of TLR9, IL-1β and TNF-α at Different Time Points. ΔC_t Values are Shown in Brackets for GpC and CpG Stimulated PBMCs with Reference to the C_t Generated by Unstimulated PBMCs

PBMCs stimulation	TLR9			IL-1β			TNF-α		
	6 hr	24 hr	48 hr	6 hr	24 hr	48 hr	6 hr	24 hr	48 hr
GpC	0.015 (6.085)	5.352 (-2.42)	0.382 (1.39)	1.376 (-0.46)	120.26 (-6.91)	1136.20 (-10.15)	0.395 (1.34)	0.003 (8.31)	0.629 (0.67)
CpG	0.057 (4.13)	6.52 (-2.71)	58.28 (-5.87)	0.768 (0.38)	43.713 (-5.45)	1323.37 (-10.37)	0.040 (4.63)	0.001 (10.95)	1260.692 (-10.3)

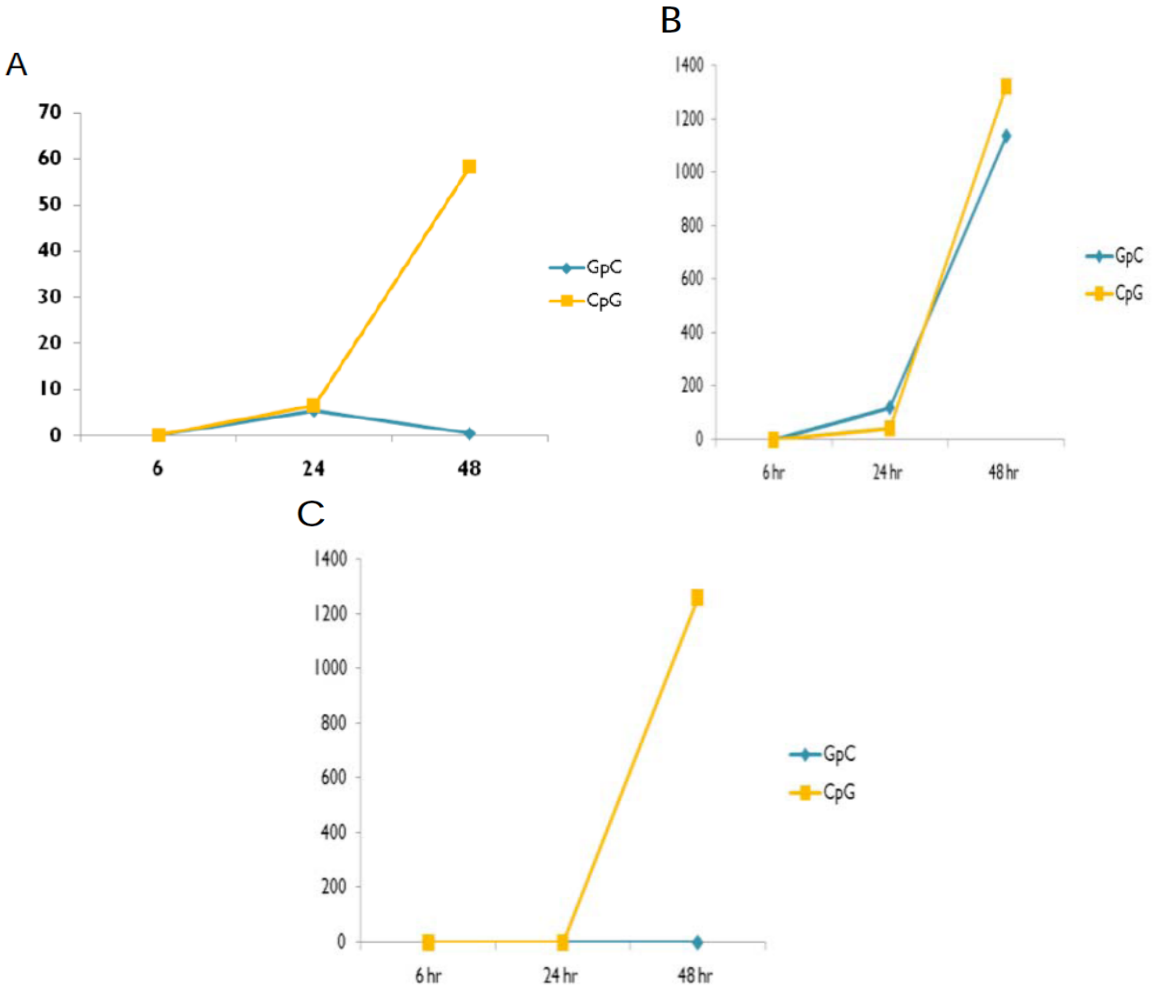

Figure 4: Relative expression analysis of TLR9 (**A**), IL-1(**B**) and TNF-α (**C**) at different time points by CpG and GpC stimulated buffalo peripheral blood mononuclear cells. Relative quantification was measured by comparing the threshold cycle (Ct) of buffalo PBMCs stimulated with CpG and GpC to the Ct generated by unstimulated PBMCs. TLR9 and cytokine expressions were normalised to GAPDH and used to calculate the relative fold difference in gene expression at 6, 12, 48 h of stimulation using the equation $2^{-\Delta\Delta}$Ct.

GpC stimulated cells did not exhibit increase in TNF-α expression compared to unstimulated PBMCs (Figure **4C**).

4. DISCUSSION

TLRs are typical type I transmembrane glycoproteins composed of an N-terminal signal peptide, an ectodomain (ECD) which mainly contains tandem leucine rich repeat (LRR) consensus motifs characterized by hydrophobic residues, a single transmembrane (TM) and a cytoplasmic region which includes a linker region, toll-interleukin 1 receptor (TIR) domain, and a C-terminal tail (T) region [12]. Toll-like receptor 9 has been the focus of considerable research attention for the ability to activate innate immune

responses, through DNA-based immunotherapeutics. TLR9 agonist CpG DNA induces B-cell proliferation, immunoglobulin production, and the secretion of a number of cytokines. Invading pathogens are recognised by toll like receptors (TLRs) present on the surface of the cells which mediate immunity. To understand the immune mechanisms, the interaction between pathogen associated molecular patterns and host defense components is essential. To address these concerns, we characterized buffalo TLR9 receptor towards N-terminal and further studied the effect of CpG, a TLR9 agonist on PBMCs of *Bubalus bubalis*.

TLR9 protein sequence of *Bubalus bubalis* in the present study was highly similar to bovine TLR9 sequences. However, these were highly dissimilar when compared to human, horse, gorilla, mouse and rat. On phylogenic analysis based on amino acid sequences the buffalo TLR9 clustered with bovines, while human, dog, sheep, goat, mice, rat and horse formed separate clades. The analysis shows close association of TLR9 proteins within bovines and high divergence with other species of animals.

TLRs bind a wide variety of pathogenic substances through their ectodomains (ECDs), which comprise 19–25 tandem copies of LRRs [13]. These LRRs are of differing size and abundance and involved in a wide variety of physiological processes. All proteins containing LRRs are thought to be involved in protein-protein interactions. TLRs contain multiple repeats that are protected by special LRR-N terminal end and LRR-C-terminal end motifs [14]. Hydrophilic conserved residue contributes towards the rigidity of the structure of LRR, on the other hand hydrophobic residues of LRRs are responsible for ligand binding [14-16]. In the present study, first LRR towards N terminal starts from Ala^{31} ends with His^{53}, second one starts from Val^{90} and ends with Leu^{109} and third is Leu^{166} to Arg^{185}. Hydrophobic nature of these amino acids residues suggests their involvement in ligand binding. The pattern of three predicted LRRs towards N terminal of buffalo TLR9 was conserved among all species except *Equus caballus* and *Rattus norvegicus* indicating substantial conservation of buffalo TLR9.

Furthermore, we analysed the pattern and kinetics of TLR9 and proinflammatory cytokines (Interleukin-1 and TNF-α production) influenced by CpG ODNs. Although there are some *in vitro* studies showing secretion of certain cytokines and chemokines following CpG-ODN exposure [17-20]. Our study investigated the effect of TLR9 ligation with CpG-ODN on proinflammatory cytokine production from buffalo PBMCs. Interleukin-1 (IL-1β) and TNF-α participate in the regulation of immune responses and inflammatory reactions. Besides their role in inflammation, IL-1β and TNF-α also attract antigen specific lymphocytes at site of infection [21]. These two cytokines are known for upregulation of adhesion molecules on endothelial cells and also upregulation of IL-6 [22]. TNF-α has also been described as a potent proinflammatory cytokine having correlation with pathogenesis [23-26]. CpG-ODN motif used herein revealed increase in proinflammatory cytokines IL-1β and TNF-α expression by buffalo PBMCs.

For elucidation of variables involved in disease resistance or susceptibility, it is necessary to characterize the components involved in immune system of the buffalo that account for the development of protective immunity. Single nucleotide polymorphims in TLR genes have been linked to susceptibility to diseases. Rapid progression of HIV-1 infection has been found to be associated with *TLR9* polymorphisms in human [27]. In the present study, the identified SNPs revealed no clinical association. This may be due to the limited disease association studies of TLR9 in domestic animals and non availability of relevant data in the database. Previously we have shown differences in relative abundance of TLR9 in buffalo tissues [28]. Higher expression of TLR9 was exhibited by lymphoid tissues as compared to PBMCs, lungs and the intestine. Further work regarding the sensitivity with which TLR9 recognizes its target and its association with buffalo diseases need to be explored. The characterization and functional analysis of buffalo TLR9 in this study may provide the first step to understand the buffalo TLR9 system.

ACKNOWLEDGEMENT/GRANT SUPPORT

The financial support from Department of Science and Technology, Government of India in the form of research project on ‘Role of TLR9 in CpG immunomodulation in buffalo calves’ is gratefully acknowledged.

REFERENCES

[1] Leulier F, Lemaitre B. Toll-like receptors--taking an evolutionary approach. Rev Nat Rev Genet 2008; 9 (3): 165-78. http://dx.doi.org/10.1038/nrg2303

[2] Temperley ND, Berlin S, Paton IR, Griffin DK, Burt DW. Evolution of the chicken toll-like receptor gene family: a story of gene gain and gene loss. BMC Genomics 2008; 9: 62. http://dx.doi.org/10.1186/1471-2164-9-62

[3] Brownlie R, Zhu J, Allan B, Mutwiri GK, Babiuk LA, Potter A, Griebel P. Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. Mol Immunol 2009; 46(15): 3163-70. http://dx.doi.org/10.1016/j.molimm.2009.06.002

[4] Krieg AM. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol 2002; 20: 709-60. http://dx.doi.org/10.1146/annurev.immunol.20.100301.064842

[5] Maletto B, Ropolo A, Moron V, Pistoresi-Palencia MC. CpG-DNA stimulates cellular and humoral immunity and promotes Th1 differentiation in aged BALB/c mice. J Leukoc Biol 2002; 72: 447-54.

[6] Mutwiri G, Pontarollo R, Babiuk S, Griebel P, van Drunen Littel-van den Hurk S, Mena A, *et al*. Biological activity of immunostimulatory CpG DNA motifs in domestic animals. Vet Immunol Immunopathol 2003; 91: 89-103. http://dx.doi.org/10.1016/S0165-2427(02)00246-5

[7] Griebel PJ, Brownlie R, Manuja A, Nichani A, Mookherjee N, Popowych Y, *et al*. Bovine toll-like receptor 9: a comparative analysis of molecular structure, function and expression. Vet Immunol Immunopathol 2005; 108(1-2): 11-6. http://dx.doi.org/10.1016/j.vetimm.2005.07.012

[8] Peter ME, Kubarenko AV, Weber AN, Dalpke AH. Identification of an N-terminal recognition site in TLR9 that contributes to CpG-DNA-mediated receptor activation. J Immunol 2009; 182: 7690-7. http://dx.doi.org/10.4049/jimmunol.0900819

[9] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 2007; 24: 1596-19. http://dx.doi.org/10.1093/molbev/msm092

[10] McGuire K, Manuja A, Russell G, Springbett A, Cragimile SC, Nichani AK, *et al*. Quantitative analysis of proinflammatory cytokine mRNA expression in *Theileria annulata*-infected cell lines derived from resistant and susceptible cattle. Vet Immunol Immunopathol 2004; 99(1-2): 87-98. http://dx.doi.org/10.1016/j.vetimm.2004.01.003

[11] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29(9): e45. http://dx.doi.org/10.1093/nar/29.9.e45

[12] West PA, Koblansky AA, Ghosh S. Recognition and signaling by toll-like receptors. Annu Rev Cell Dev Biol 2006; 22: 409-37. http://dx.doi.org/10.1146/annurev.cellbio.21.122303.115827

[13] Bell JK, Mullen GED, Leifer A, Mazzoni A, Davies DR, Segal DM. Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends Immunol 2003; 24: 528-53. http://dx.doi.org/10.1016/S1471-4906(03)00242-4

[14] Matsushima N, Tanaka T, Enkhbayar P, Mikami T, Taga M, Yamada K, *et al*. Comparative sequence analysis of leucine-rich repeats (LRRs) within vertebrate toll-like receptors. BMC Genomics 2007; 8: 124. http://dx.doi.org/10.1186/1471-2164-8-124

[15] Bella J, Hindle KL, McEwan PA, Lovell SC. The leucine-rich repeat structure. Cell Mol Life Sci 2008; 65: 2307-33. http://dx.doi.org/10.1007/s00018-008-8019-0

[16] Werling D, Jann OC, Offord V, Glass EJ, Coffey TJ. Variation matters: TLR structure and species-specific pathogen recognition. Trends Immunol 2009; 30: 124-30. http://dx.doi.org/10.1016/j.it.2008.12.001

[17] Zhao Q, Temsamani J, Zhou RZ, Agrawal S. Pattern and kinetics of cytokine production following administration of phosphorothioate oligonucleotides in mice. Antisense Nucleic Acid Drug Dev 1997; 7(5): 495-502. http://dx.doi.org/10.1089/oli.1.1997.7.495

[18] Qiao B, Li B, Yang X, Zhang H, Chu Y, Wang Y. Specific siRNA downregulated TLR9 and altered cytokine expression pattern in macrophage after CpG DNA stimulation. Cell Mol Immunol 2005; 2: 130-5.

[19] Hacker H, Redecke V, Blagoev B, Kratchmarova I, Hsu LC, Wang GG. Specificity in toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. Nature 2006; 439: 204-7. http://dx.doi.org/10.1038/nature04369

[20] Doreswamy R, Saini M, Swarup D, Singh VK, Upreti S, Das A, *et al*. Interferon alpha characterization and its comparative expression in PBM cells of *Capra hircus* and *Antelope cervicapra* cultured in the presence of TLR9 agonist. Mol Biol Int 2010. http://dx.doi.org/10.4061/2010/573426

[21] Medzitov K, Janeway C. Innate immunity: impact on the adaptive immune response. Curr Opin Immunol 1997; 9: 4-9. http://dx.doi.org/10.1016/S0952-7915(97)80152-5

[22] Kandimalla ER, Zhu FG, Bhagat L, Yu D, Agarwal S. Toll-like receptor 9: modulation of recognition and cytokine induction by novel synthetic CpG DNAs. Biochem Soc Trans 2003; 31(3): 654-48. http://dx.doi.org/10.1042/BST0310654

[23] Bielefeldt OH, Campos M, Snider M, Raipin N, Beshkorwayne T, Popowych Y, *et al*. Effect of chronic administration of recombinant bovine tumour necrosis factor to cattle. Vet Pathol 1989; 26: 462-72. http://dx.doi.org/10.1177/030098588902600602

[24] Rieckmann P, Michel U, Albrecht M, Brück W, Wöckel L, Felgenhauer K. Soluble forms of intercellular adhesion molecule-1 (ICAM-1) block lymphocyte attachment to cerebral endothelial cells. J Neuroimmunol 1995; 60(1-2): 9-15. http://dx.doi.org/10.1016/0165-5728(95)00047-6

[25] Manuja A, Malhotra DV, Sikka VK, Sangwan AK, Kumar B, Nichani AK. *Theileria annulata* infected cell lines from different parts of India induce variable cytokine gene expression. Online J Vet Res 2003; 7: 71-7.

[26] Manuja A, Malhotra DV, Sikka VK, Sangwan AK, Sharma R, Kumar B, *et al*. Isolates of *Theileria annulata* collected from different parts of India show phenotypic and genetic diversity. Vet Parasitol 2006; 147: 242-52. http://dx.doi.org/10.1016/j.vetpar.2006.01.021

[27] Bochud PY, Hersberger M, Taffé P, Bochud M, Stein CM, Rodrigues SD, *et al*. Polymorphisms in Toll-like receptor 9 influence the clinical course of HIV-1 infection. AIDS 2007; 21(4): 441-6. http://dx.doi.org/10.1097/QAD.0b013e328012b8ac

[28] Manuja A, Manuja BK, Dhingra M, Sarkar S. Differential expression of toll-like receptor 9 by various immune compartments of buffalo. Indian J Anim Sci 2012; 82(4): 427-9.

Received on 17-01-2013 Accepted on 20-04-2013 Published on 18-06-2013

DOI: http://dx.doi.org/10.6000/1927-520X.2013.02.02.2