EXPRESSION AND STRUCTURAL VARIATIONS OF TOLL-LIKE RECEPTOR 4 GENE IN BUFFALO AND CATTLE

Rajendran Lakshmi[1,*], Jacob Thanislass[2] and Balanehru Subramanian[3]

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ABSTRACT

Systematic studies on expression and characterization of toll like receptors (TLRs) in buffalo and cattle will help to understand the innate immune response specific to each species of animals. TLR4 gene is one of the most studied innate immune receptors which is mainly triggered by lipopolysaccharide. Therefore, the present study was carried out to investigate the mRNA expression and sequence analysis of TLR4 in buffalo in comparison to that of cattle. *In vitro* expression of TLR4 was assessed by challenging the Peripheral Blood Mononuclear Cells (PBMC) with bacterial LPS which showed significant increase of TLR4 gene expression ($P \leq 0.01$) in buffaloes when compared to cattle. Further the comparison of mRNA sequence of TLR4 of buffalo and cattle revealed 96% similarity with the cattle sequence. The primary structure of protein showed the highest percent of leucine amino acid for both the species of animals. At the same time, the number of Leucine Rich repeats (LRR) of buffalo is varied from those present in cattle TLR4. Alpha helix is the prominent secondary structure, however, there were few amino acid variations between buffalo and cattle in certain regions of TLR4 which altered the three dimensional conformation of TLR4 proteins, this in-turn possibly can influence the binding affinity and interaction with pathogens which may influence the difference in immune response in buffalo when compared to that of cattle.

Keywords: *Bubalus bubalis*, buffaloes, innate immunity, TLR4, LPS, cattle, gene

INTRODUCTION

Livestock play an important role in the rural source of income and economy of developing and several developed countries. They are providers of nutriment for billions of rural and urban households as well as income and employment for producers and others working in simple and sometimes complex value chains. Domestic water buffalo (*Bubalus bubalis*) is an important livestock species in tropical countries because it is a better

[1]Department of Veterinary Physiology and Biochemistry (Veterinary College and Research Institute, Salem), Tamil Nadu Veterinary and Animal Sciences University, Chennai, India, *E-mail: lakshmivetbio@gmail.com

[2]Department of Veterinary Biochemistry, Rajiv Gandhi Institute of Veterinary Education and Research, Puducherry, India

[3]Central Inter-Disciplinary Research Facility (CIDRF), Sri Balaji Vidyapeeth, Puducherry, India

source of high-quality milk and meat, leather and traction power which can survive on marginal land and subsist on low-quality forages (Ali *et al*., 2017; Dos Santos *et al*., 2017; Nardi Junior *et al*., 2012). Water buffalo and cattle exposed to a similar spectrum of infectious agents, but water buffalo found to respond differently to certain bacterial and parasitic infections (Borriello *et al*., 2006). But, the management of buffaloes, including health management is the same as that of cattle which may not find answers to all the problems of diseases of buffaloes. The innate immune system is essential for the host defense and responsible for the early detection of invading pathogens. Effectors mechanism of the innate immune system activated immediately after infection. There are 11 TLRs known in mammals (Rabindra and Akira, 2005), and they differentially expressed in several types of cells including the immune cells, epithelial cells, endothelial cells, astrocytes, endometrium, etc. TLR expression patterns have been determined in humans (Zarember and Godowski, 2002), mice (Prueet *et al*., 2004), chickens (Iqbal *et al*., 2005) and bovines and ovines (Menzies and Ingham, 2006). Higher expression of TLRs reflects a better innate immune response and disease resistance. TLR4 gene is foremost innate immune receptors activated by lipopolysaccharide (LPS). TLR4 activation by LPS leads to the secretion of pro-inflammatory chemokines and cytokines (Ibeagha-Awemu *et al*., 2008). Buffalo is an animal of great economic importance in India, but the understanding of innate immune response specific to buffaloes is scanty. Characterization and expression of toll-like receptors will be one of the approaches to understand the underlying principle of innate immune responses. Therefore, the study is devised to understand the innate immune system concerning TLR4 of buffalo in comparison to that of cattle.

MATERIALS AND METHODS

Ethical approval

The study was approved by the institutional animal ethics committee of Pondicherry University (PU/CAHF/AH/19th IAEC/2017/01). Adequate measures were taken to minimize pain or discomfort following the international animal ethics committee.

Sample collection

Blood samples were collected from apparently healthy animals of Murrah buffalo and Jersy crossbred cattle (each six in number) from the private cattle farms located in Puducherry, India. Blood samples were collected from the jugular vein of buffalo and cattle, using sterile needles and syringes with 0.5 ml of acid citrate dextrose anticoagulant for 5 ml of blood.

Isolation of peripheral blood mononuclear cells (PBMCs)

The blood was slowly layered over on an equal volume of HiSEP (Himedia) with specific gravity 1.077 g/ml. It was then centrifuged at 600 g for 30 minutes and then the interface containing the PBMCs was collected and washed twice in sterile phosphate buffered saline (PBS).

PBMCs culture and stimulation with LPS

Buffalo and cattle PBMCs were incubated in 5 ml of sterile RPMI-1640 medium (with L Glutamine) taken in tissue culture flasks in a CO_2 incubator (5 percent CO_2 pressure) at 37ºC (80 percent RH) for 3 h. After incubation, the medium was discarded along with the dead floating cells.

The cells which were anchored, mostly monocytes, and found to be live were used for further study.

PBMC were subsequently induced with LPS (Sigma) at a concentration of 15 µg/ml by incubating in RPMI 1640 medium for 2 h in a CO_2 incubator (5% CO_2 pressure) at 37ºC (80% RH). Similar preparation of cells was subjected to incubation without the LPS, which served as control. After the incubation with LPS, the cells were harvested by centrifugation and were used for TLR4 gene expression study.

Isolation of Total RNA and cDNA synthesis

Total RNA from PBMC cells was isolates with the TRIzolPlus-RNA purification kit (Sigma). Using agarose gel electrophoresis, the RNA quality was determined. DNA residual in the extracted total RNA was removed by DNase1 treatment. The quantity of RNA isolated was measured by NanoDrop Spectrophotometer (Thermo Scientific, USA).

Complementary DNA was synthesized from isolated RNA using RevertAid first strand cDNA synthesis kit (Thermo Scientific, K1622). The reactions were set up in 0.2 ml PCR tubes. Template RNA 5 µg, Oligo (dT) 18 primer 1.0 µl, 5X reaction buffer 4.0 µl, Ribolock 1 µl, dNTP mix (10 mM) 2.0 µl, Revertaid 1.0 µl and Nuclease free water. The contents of the tube were mixed gently and centrifuged briefly. For qRT PCR works, oligo (dT) 18 primer were used for cDNA synthesis, the reaction mix was incubated for 60 minutes at 42°C. The reaction was terminated at 70°C for 5 minutes.

Primers synthesis

Primers for RT-qPCR of TLR4 and β-actin (internal control gene) were designed from published bovine mRNA sequences available from GenBank. Primers were designed using Primer3 software (Table 1). The primers designed were custom synthesized from Sigma Aldrich and were diluted to a concentration of 10 pM/µl.

PCR standardization and RT-qPCR

The PCR was carried out in a volume of 12.5 µl in 0.2 ml PCR tubes. The PCR was standardized for different gradients of temperatures. For qPCR analysis, MaximaSYBR Green Master Mix (2X) with ROX (Thermo Scientific) were used and RT-qPCR reactions were performed using real time PCR system (ECO software). The RT-qPCR mix was in a final quantity of 20 µL consist of 12.5 µl of Maxima syber green master mix, 0.5 µl each of forward and reverse primer, 1 µl of Template cDNA and 5.5 µl of nuclease free water. The thermal profile included initial denaturation at 95°C for10 minutes, succeed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 20 seconds, extension at 72°C for 30 seconds. Dissociation (melt) curve analysis was done after each PCR. The protocol for melt curve analysis was 95°C for 15 seconds, 55°C for 15 seconds followed by 95°C for 15 seconds. Data acquisition was performed during the final denaturation step. The gene expression levels were analyzed with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Sequencing of amplified product

Primers used for amplification of TLR4 genes were designed in three sets, using online software Primer 3 utilizing the TLR4 sequence (HQ343416.1). The conditions for PCR were optimized for annealing temperature using gradient PCR and the annealing temperature showing efficient amplification with the absence of non-specific products was selected (Table 2). The amplified product of forward and reverse fragments was sequenced at Eurofins Genomics

Pvt. Ltd, Bangalore, India.

Statistical analysis

Tukey's HSD was applied to test the significance of TLR4 gene expression between buffalo and cattle using R software.

RESULTS AND DISCUSSIONS

For successful adoption of health management strategies and to address problems unique to water buffaloes it is necessary to characterize the components of the immune system and to elucidate the features in the immune response that account for the development of protective immunity. TLR4 is an extracellular receptor and implicated in the recognition of Gram-negative bacterial components, lipopolysaccharide (Bi *et al*., 2018). In the host system, LPS induction is facilitated by lipopolysaccharide-binding protein and TLR4 receptor. The experiments on human and mice cell lines have found that TLR4 recognized the LPS specifically (Lamping *et al*., 1998; Poltorak *et al*., 1998; Hoshino *et al*., 1999; Takeda and Akira, 2005). There is no work reported on the expression profiles of LPS induced TLRs in buffalo. Since the major diseases of buffalo and cattle are caused by Gram-negative bacteria, understanding the LPS induced TLR4 expression is necessary to assess the immune responses in buffalo.

As previously reported in literature PBMC from crossbred cattle challenged with LPS showed a statistically significant increase in mRNA expression of TLR4 when compared to the control group (Panigrahi *et al*., 2014). Available references also reveals that bovine mammary tissue after experimental *in vivo* infection with *E. coli* (Petzl *et al*., 2008) and mammary epithelial cells of cattle treated with *E. coli* bacteria (Griesbeck-Zilch *et al*., 2008), resulted in up-regulation of TLR4 gene by producing proinflammatory cytokines required to combat invading pathogens. In our study, when comparisons were made for the TLR4 mRNA level of expression between buffalo and cattle along with TLR2 and TLR3 genes, interestingly buffaloes showed significantly ($P \leq 0.01$) higher expression of TLR4 gene when compared to cattle (Table 3 and Figure 1). As suggested by the report of Vigensha *et al.* (2012), this difference recapitulates the species wise differences of such gene expression levels. Further this difference reflects the inherent capacity of the cells derived from buffaloes to mount higher immune response when exposed to specific ligand where both the species of animals are reared in the same geographical area with similar management strategies. Besides, the difference may be due to the fact that the role of miRNA binding to the 3′UTR of the gene in the fine-tuning gene expression (Benakanakere *et al*., 2009), which could play a role in the difference in regulation of gene expression in buffalo and cattle.

Further we analyzed cDNA nucleotide sequences of TLR4 and *in silico* translated amino acid sequences of buffalo in comparison to that of cattle. The full-length coding sequence of TLR4 cDNA of buffalo (Accession No. MT424002) and cattle (Accession No. MT424003) were submitted to GenBank. The percent nucleotide and amino acid identity of the buffalo TLR4 were compared with a few other ruminant species. There was a high degree of similarity with cattle (96%) and 94% similarity with goat and sheep sequences. At the level of amino acid, 96% identity was observed with cattle and sheep, while with goat 90% similarity was observed. SMART was used to predict the ecto, transmembrane, and TIR domains using the deduced amino acid sequences of buffalo and

cattle TLR4. There is a difference in the number of LRR in buffalo and cattle sequence, the number of LRR of TLR4 detected is 11 and 12, respectively. The primary structure predicted using Protean DNA STAR revealed the presence of leucine and serine amino acids in the major portions of TLR4 of buffalo and cattle. Alpha helix is the prominent secondary structure predicted (PSI PRED) in both the species. But there are few amino acid variations observed in buffalo, specifically at 270, 419 and 512th position of the polypeptide which favored coiled coil-like structure but in case of cattle, the amino acids present favored the helical like structure (Table 4) which could account for the difference seen in the tertiary structure of the protein (Figure 2).

Tertiary structure obtained from SWISS-MODEL for TLR4 protein, revealed a distinctive horseshoe-shaped structure, this structure was formed by the "leucine-rich repeats. The number and structure of the LRR of TLR have a significant effect on the function of the pathogen recognition receptor system (Dubey *et al*., 2013). Although it is considered that the ligands for each TLR are similar, the difference in their structure may point to the fact that the TLRs in different species could bind to ligands differently or that the TLR ligand interaction may not induce similar types of response in different species. Therefore it can be construed that the structural variations observed in TLR4 of buffalo may influence the binding affinity and interaction with the pathogen to boost the innate host immune response in buffalo differently when compared to cattle. Earlier studies in our laboratory also demonstrated difference in TLR signaling in water buffaloes (Thanislass *et al*., 2009; Nisha *et al*., 2013).

Understanding the fact that genetic disease resistance is a multigene trait and increased production of one of few immune particles may or may not fully explain the underlying mechanism of immunity of the individual animal, but based on the evidence generated in this study, it can still be considered that higher levels of TLR4 mRNA expression and secondary structural variation observed in buffalo could be one of the factors may explain the difference in the kind of immunity observed in buffalo. The present study has been limited only to the quantification of TLR4 mRNA and structural variations; hence further studies are required to ascertain their functional significance through the comparison of the downstream cytokines induced by TLR4 ligands on the PBMCs of these species.

CONCLUSION

This study reveals the higher expression and structural variation of the TLR4 gene of buffalo when compared to cattle that may play a key role in the innate immune system by recognizing the invading pathogens to boost the innate host disease resistance in buffalo. The knowledge generated in this study will help us to recognize important genetic alleles associated with disease resistance specific to buffaloes.

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Table 1. Primer sequence designed in three sets for amplification and sequencing of cDNA of TLR4 gene in buffalo and cattle.

Primer information		Sequences (5'→3')	Annealing temperature	Product size
SET I	Forward	GCAGGGGAGAGACGACACTA	56.6ºC	959 bp
	Reverse	CAATGGTCAGGTTGCACAGT		
SET II	Forward	TCAAATGCCCCTACTCAACC	56.6ºC	1238 bp
	Reverse	GCTCTGCACACATCATTTGC		
SET III	Forward	CCTTCAGGTGCTGAATATGAGTC	58.5ºC	1021 bp
	Reverse	TAACTGAACACGCCCTGCAT		

Table 2. Primer sequence designed for TLR4 and beta actin genes for RT-PCR assay.

Primer information		Sequences (5'→3')	Annealing temperature	Product size
TLR4	Forward	CTTGCGTACAGGTTGTTCCTAA	62ºC	153
	Reverse	CTGGGAAGCTGGAGAAGTTATG		
Beta Actin	Forward	ATCGGCAATGAGCGGTTCC	62ºC	143
	Reverse	GTGTTGGCGTAGAGGTCCTTG		

Table 3. Comparison of relative expression of TLR4 gene with other TLRs in cattle and buffalo.

Gene	Animal	$\Delta C_{q\ Mean}$	ΔΔCq	Fold change
TLR2	Cattle	7.27±0.205	-1.58	2.99^{a*}
	Buffalo	16.12±0.373	-2.12	4.35^{a**b*}
TLR3	Cattle	1.86±0.080	-1.04	2.06^{a*}
	Buffalo	17.98±0.157	-1.76	3.39^{a*}
TLR4	Cattle	2.25±0.234	-0.41	1.33
	Buffalo	6.19±0.144	-2.54	5.80^{a**b**}

Statistical significance at *P≤0.01; * P≤0.05 compared with [a] control and [b]cattle.

Table 4. Variation in amino acid position and secondary structure of TLR4 protein in buffalo and cattle.

Amino acid position		Buffalo		Cattle
Amino acid change		Secondary structure	Amino acid change	Secondary structure
270	K	Coiled	N	Helical
419	V	Coiled	M	Helical
512	S	Coiled	A	Helical

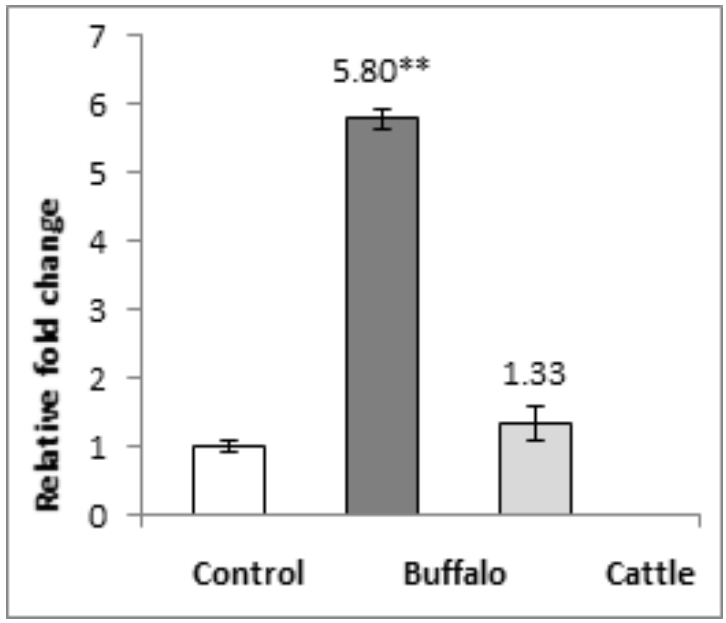

Figure. 1 mRNA expression of TLR4 gene expressed as relative fold change in buffalo and cattle (statistical significance at **P≤0.01 compared with control and cattle).

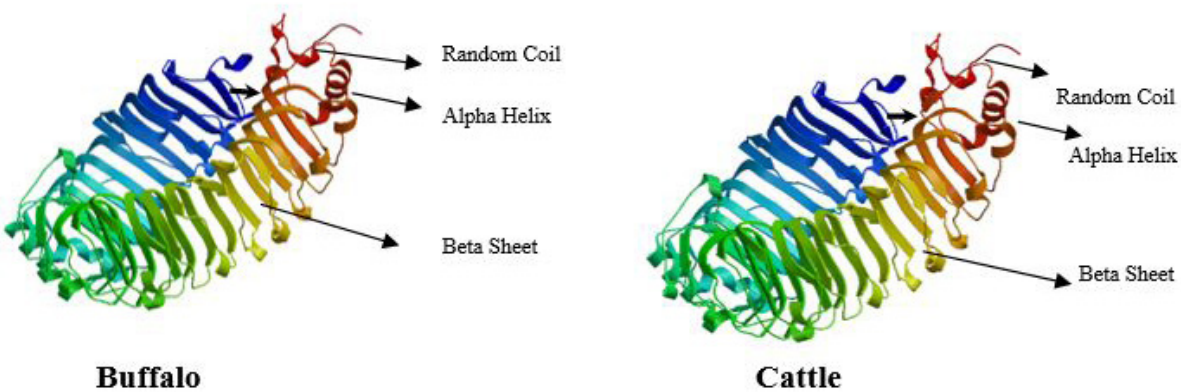

Figure 2. Structural variation of TLR4 protein in buffalo and cattle.

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REFERENCES

Ali, S., Q. Ali, F. Melzer, I. Khan, A. Shamin, H. Neubauer and S.M. Jamal. 2013. Isolation and identification of bovine *Brucella isolates* from Pakistan by biochemical test and PCR. *Trop. Anim. Health Pro*., **46**(1): 73-78. DOI: 10.1007/s11250.013-0448.6

Benakanakere, M.R., Q. Li, M.A. Eskan, A.V. Singh, J. Zhao, J.C. Galicia, P. Stathopoulou, T.B. Knudsen and D.F. Kinane. 2009. Modulation of TLR2 protein expression by miR-105 in human oral keratinocytes. *J. Biol. Chem*., **284**(34): 23107-23115. DOI: 10.1074/jbc.M109.013862.

Bi, D., Y. Wang, Y. Gao, X. Li, Q. Chu, J. Cui and T. Xu. 2018. Recognition of lipopolysaccharide and activation of NF-κB by cytosolic sensor NOD1 in Teleost fish. *Front. Immunol*., **9**: 1413. DOI: 10.3389/fimmu.2018.01413.

Borrielo, G., R. Capparelli, M. Bianco, D. Fenizia, F. Alfano, F. Capuano, D. Ercolini, A. Parisi and S. Roperto. 2006. Genetic resistance to *Brucella abortus* in the Water buffalo (*Bubalus bubalis*). *Infect. Immun*., **74**(4): 2115-2120. DOI: 10.1128/IAI.74.4.2115–2120.2006

Dos Santos, L.S., J.C. Sa, D.L. Dos Santos Ribeiro, N.P. Chaves, J.P. da Silva Mol, R.L. Santos, T.A. da Paixao and A.V.de Carvalho Neta. 2017. Detection of *Brucella sp.* infection through serological, microbiological, and molecular methods applied to buffaloes in Maranhao state, Brazil. *Trop. Anim. Health Pro*., **49**(4): 675-679. DOI: 10.1007/s11250-017-1238-3

Dubey, P.K., S. Goyal, P. Kathiravan, B.P Mishra, S.K. Gahlawat and R.S. Kataria. 2013. Sequence characterization of River buffalo Toll-like receptor genes 1-10 reveals distinct relationship with cattle and sheep. *Int. J. Immunogenet*., **40**(2): 140-148. DOI: 10.1111/j.1744- 313X.2012.01135.x

Griesbeck-Zilch, B., H.H.D. Meyer, C. Kühn, M. Schwerin and O. Wellnitz. 2008. Staphylococcus aureus and Escherichia coli cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells. *J. Dairy. Sci*., **91**(6): 2215-2224. DOI: 10.3168/jds.2007-0752.

Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR 4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR 4 as the LPS gene product. *J. Immunol*., **162**(7): 3749-3752. Available on: http://www.jimmunol.org/content/162/7/3749

Ibeagha-Awemu, E.M., J.W. Lee, A.E. Ibeagha, D.D. Bannerman, M.J. Paape and X. Zhao. 2008. Bacterial lipopolysaccharide induces increased expression of Toll-like receptor (TLR) 4 and downstream TLR signaling molecules in bovine mammary epithelial cells. *Vet. Res*., **39**(2): 11. DOI: 10.1051/vetres:2007047

Iqbal, M. and V.J. Philbin. 2005. Expression patterns of chicken Toll-like receptor mRNA in tissues, immune cell subset and cell lines. *Vet. Immunol. Immunopathol*., **104**(1-2): 117-127. DOI: 10.1016/j.vetimm.2004.11.003.

Lamping, N., R. Dettmer, N.W. Schroder, D. Pfeil, W. Hallatschek, R. Burger and R.R.

Schumann. 1998. LPS-binding protein protects mice from septic shock caused by LPS or gram-negative bacteria. *J. Clin. Invest*., **101**(10): 2065-2071. DOI: 10.1172/ JCI2338

Livak, K.J. and T.W. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. *Methods*, **25**(4): 402-408. DOI: 10.1006/meth.2001.1262

Menzies, M. and A. Ingham. 2006. Identification and expression of Toll-like receptors 1-10 in selected bovine and ovine tissues. *Vet. Immunol. Immunop*., **109**(1-2): 23-30. DOI: 10.1016/j.vetimm.2005.06.014.

Nardi, G.J., M.G. Ribeiro, A.M. Jorge, J. Megid and L.M.P. Silva. 2012. Serological profile of buffalo (*Bubalus bubalis*) female calves vaccinated with standard *Brucella abortus* strain 19 using rose Bengal, 2-mercaptoethanol and complement fixation test. *Biologicals*, **40**(2): 158-161. DOI: 10.1016/j.biologicals.2011.09.016.

Nisha, P., J. Thanislass, P.X. Antony, H.K. Mukhopadhyay and K.V.S. Reddy. 2013. Bacterial DNA induced TNF-α expression in buffaloes (Murrah) in comparison to that of cross breed cattle. *Journal of Buffalo Science*, **2**(1): 8-11. DOI: 10.6000/1927-520X.2013.02.01.2

Panigrahi, M., A. Sharma and B. Bhushan. 2014. Molecular characterization and expression profile of partial TLR4 gene in association to mastitis in crossbred cattle. *Anim. Biotechnol*., **25**(3): 188-199. DOI: 10.1080/10495398.2013.856797

Petzl, W., H. Zerbe, J. Günther, W. Yang, H.M. Seyfert, G. Nürnberg and H.J. Schuberth. 2008. *Escherichia coli*, but not *Staphylococcus aureus* triggers an early increased expression of factors contributing to the innate immune defense in the udder of the cow. *Vet. Res*., **39**(2): 18. DOI: 10.1051/ vetres:2007057.

Poltorak, A., X. He, I. Smirnova, M.Y. Liu, H.C. Van, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Casatagnoli, B. Layton and B. Beutler. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene. *Science*, **282**(5396): 2085-2088. DOI: 10.1126/science.282.5396.2085

Prueet, S.B., Q. Zheng, R. Fan, K. Matthews and C. Schwab. 2004. Acute exposure to ethanol affects Toll-like receptor signaling and subsequent responses: An overview of recent studies. *Alcohol*, **33**(3): 235-239. DOI: 10.1016/j.alcohol.2004.08.003

Rabindra, N.B. and S. Akira. 2005. Toll-like receptor signaling: Emerging opportunities in human diseases and medicine. *Current Immunology Reviews*, **1**(1): 81-90. DOI: 10.2174/ 1573395052952897

Takeda, K. and S. Akira. 2005. Toll-like receptors in innate immunity. *Int. Immunol*., **17**(1): 1-14. DOI: 10.1093/intimm/dxh186.

Thanislass, J., G. Yuvaraj and K.V. Subba Reddy. 2009. Characterization of TLR4 signaling in Water buffalo (*Bubalus bubalis*). *Vet. Res. Commun*., **33**(2): 97-102. DOI: 10.1007/ s11259-008-9074-6

Vignesha, A.R., S. Dhanasekarana, G.D. Raja, C. Balachandranb, N. Pazhanivelb, C. Sreekumarc, K.G. Tirumurugaana, A. Rajaa and K. Kumanana. 2012. Transcript profiling of pattern recognition receptors in a semi domesticated breed of buffalo, Toda, of India. *Vet. Immunol. Immunop*., **147**(1-2):

51-59. DOI: 10.1016/j.vetimm. 2012.02.009

Zarember, K.A and P.J. Godowski. 2002. Tissue expression of human toll-like receptors and differential regulation of toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J. Immunol*., **168**(2): 554-561. DOI: 10.4049/ J Immunol.168.2.554.