ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Genetic Variations and Protein Domain Architecture of Toll-like receptor 6 Gene in River Buffalo, Egyptian Breed.

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ABSTRACT

Water Buffalo are the main source of meat and milk in several countries. River buffalo (*Bubalus bubalis bubalis*) constitute 77% of the water buffalo population (195 million). The Egyptian buffalo breed is of the river type and their population amounts exceed 4million heads. *Toll-like receptor* genes (*TLR*s) play an important role in innate immunity. In the present study *TLR6* gene which recognizes ligands of bacteria and plays an important role in defense against invading pathogen was characterized in Egyptian buffalo breed. Its full coding sequence and the protein architecture domains were analyzed and were compared with other river buffalo breeds and with cattle, sheep and goat. Thirteen genetic variations were reported, for the first time in *TLR6* coding region of river buffalo, six of which were non-synonymous SNPs (nsSNPs). One nsSNP (I15V) was found in the transmembrane domain that plays a main role in signal transduction. Three nsSNPs (N173S, I507V, Q519R) were in LRRs which are responsible for ligand recognition and may cause change in responsiveness. Two nsSNPs (L685V and H713P) were in TIR domain that may affect signal transduction. H713P was found intolerable causing potential functional significance.

Keywords: Egyptian Buffalo, *Toll-like receptor 6*, polymorphism, protein architecture domains, Genetic variations.

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ISSN: 0975-8585

INTRODUCTION

Worldwide water buffalo population (195 million heads) includes both river (2n=50) and swamp (2n=48) buffaloes, 77% of which are river buffalo [1]. Egypt ranks the third after India and Pakistan [1] in river buffalo population. The Egyptian buffalo population is around 4 million heads. Buffalo plays a main role in agricultural economy of many countries. Buffalo are of great economic potential and it is looked upon as the most important tropical bovine species [2]. They are main source of milk and meat. Buffalo were reported to have more resistance than cattle [3].

Innate immunity has been found responsible for the protection of the host against any invasion. It uses cells such as phagocytic cells, inflammatory mediators-releasing cells, natural killer cells, molecular components like complement, cytokines and acute phase proteins [4]. Innate immune system in mammals provides host defense against a variety of pathogens without previous exposure [5]. *Toll-like receptor* genes (*TLR*s) play an important role in innate immunity. They are considered pattern-recognition receptors which recognize pathogen-associated molecular patterns which are vital products of the microorganisms not present in eukaryotic cells. Association between innate immune gene variation and differential susceptibility to diseases has been reported [6, 7]. *TLR*s are mainly expressed on antigen presenting cells such as macrophages and B-lymphocytes [5]. They are present in eukaryotes such as plants [8] and metazoan [9]. In mammals up to 13 *TLR*s have been found, 10 of which have been assigned and mapped in cattle [10].

The mammalian *TLR*s are type I transmembrane proteins of the interleukin-1 receptor family that possess N-terminal leucine-rich repeats (LRR) involved in ligand recognition and subsequently the signaling pathway [11], transmembrane domains, Carboxy-terminal LRR (LRRCT) domain and an intracellular Toll/IL-1R (TIR) homologous domain for signal transduction [5]. Each *TLR* is capable of recognizing a certain type of ligand through which it can recognize a pathogenic invasion. Some recognize ligands of bacteria such as *TLR*s *1,2,4,5,6,9,11* while others recognize viral ligands such as *TLR*s *2,3,4,7,8,9* [12].

Identifying genetic variations in protein domains of *TLR* gene can seriously affect its function which has a direct impact on quantitative trait of economic importance. Variations in LRR domains may cause changes in responsiveness towards a foreign pathogenic or non-pathogenic microorganism [13]. Whereas variations in transmembrane domain plays a main role in *TLR* ligand recognition and signal transduction [14]. Genetic variants in TIR domain may affect the TIR role in signal transduction that affects the inflammatory response and the response to invading pathogens [11].

TLR6 gene is present in a cluster with *TLR1* and *TLR10* in several species. This cluster is present on bovine chromosome BTA6 within a region of about 50Mb [15] whereas in river buffalo it is located on chromosome number 7 (BBU7), the homologue of BTA 6 [16]. *TLR*s genes protein architecture domain, have been recently investigated in buffalo. Protein architecture domains of *TLR1-10* genes in Nili-Ravi (Indian buffalo breed) was investigated [17] whereas *TLR2-9,* except *TLR6* were investigated, in 6 other different breeds [18].

The aim of the present study was to identify the genetic variations *in TLR6* coding region of Egyptian buffalo breed and detect their effect on ligand recognition and signal transduction by characterizing its protein architecture domains. Comparison between Egyptian buffalo and other buffalo breeds, cattle, sheep and goat were investigated.

MATERIALS AND METHODS

Genomic DNA extraction

Ten blood samples of unrelated healthy buffalo were collected from a private farm "United Farms Group Company" by their specialized veterinarian. Genomic DNA was extracted from leukocytes by salting out method according to Miller *et al* [19]. The DNAs concentrations were measured using Nanodrop 1000 (Thermoscientific) and used as a template in Polymerase chain reaction (PCR).

Primers design

Three overlapping primer pairs were designed to cover *TLR6* full coding region Table (1). Primers were designed using Primer3 software [20]. The primers were tested in Oligo Analyzer program ver. 1.0.3 to ensure their specificity. They were synthesized by Amersham Pharmacia Biotech.

Table (1): Primers pairs used for *TLR6*

Primer	5'-sequence-3'	Target length	Annealing temperature	Accession no.
F1	ATCAGCAGCAACCCTCCGGG	1532 bp	61.1 ºC	HQ327992.1
R1	AGGCTGCTAAAGATACCACACCCA			HQ327992.1
F2	TCTTGGGTTGGGAGTATAG	860 bp	50.9 ºC	AC_000163.1
R2	CATTCGCTCTGGACAAAGTTG			AC_000163.1
F3	CTGCCTGGGTGAAGAATGAA	940 bp	53.5 ºC	AC_000163.1
R3	CTGGGGCCTGAAAAGACATA			AC_000163.1

Polymerase Chain Reaction and Sequencing

Each amplification reaction (25µl) contained 100ng of buffalo DNA, 0.2 mM dNTPs (Finnzymes), 1XDreamTaq™ Green buffer (Fermentas), 1.0 µM Forward and reverse primers, 1.25 units DreamTaq™ Green DNA polymerase (Fermentas). The reaction mixture was run in a Q-Cycler, HVD LifeSciences. The following cycling conditions were used: 3 min. at 94 ºC; 35 cycles for 1 min at 94 ºC; 2 min annealing; 2 min at 72 ºC and a final extension for 10 min at 72 ºC. The PCR products were electrophoresed on 1.5% agarose gel with ethidium bromide (Applichem). The gels were inspected by UV light using Gel documentation system (In Genius, Syngenebioimaging).

Larger amounts of PCR products were produced to allow excision from the gel. The PCR products were purified using MEGAquick-spin™ Total Fragment DNA Purification Kit (iNtRON biotechnology) after being excised from the gel according to the kit's instructions. Each amplicon was sequenced using Sanger method by Macrogen, using reverse and forward primers. For each sample the nucleotides sequence of full coding region was extracted from its three overlapped fragments.

Sequence analysis

All PCR amplicons from the 10 buffalo were sequenced and the full 10 *TLR6* coding region were extracted and examined. Polymorphic sites were determined by visual examination of sequence's charts. The protein sequence was identified using Open Reading Frame (ORF) (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The amino acids substitutions due to the presence of the SNPs were determined and their tolerance were examined and analyzed using Software Implemented Fault Tolerance "SIFT" tool [21].

Characterization of the protein architecture domains

TLR6 protein domain architecture was determined using SignalP 4.1 [22] for detecting the presence of signal peptide, whereas the transmembrane regions (TM); Leucine-rich repeats (LRR); LRR C-terminal (LRR-CT), N-terminal (LRR-NT) and Toll/IL-1 receptor (TIR) were predicted via SMART [23]. LRRfinder [24] was used to predict other LRRs which were not identified by SMART.

ISSN: 0975-8585

RESULTS AND DISCUSSION

*Toll-like receptor*s are the most important component of pathogen receptor recognition system and their characterizations are essential to evaluate the innate immune system. *TLR6* gene belong to *TLR1*-family which also includes *TLR1*,*TLR2* and *TLR10* [25]. River buffalo has been known to be less susceptible to diseases than cattle [26]. They are mainly present in India, Pakistan, Egypt and Italy. During the last decades river buffalo has been introduced to the Americas [27].

Table (2): Nucleotide and amino acid variations in Egyptian buffalo breed *TLR6* coding region

Nucleotide position	Nucleotide variation	Amino acid variation	Position of a.a. variation	Predicted Architecture Domains		Side-chain Polarity changes
				Via SMART	Via LRRfinder	
43	A/G	I /V	15	Transmembrane	Transmembrane	Nonpolar/ Nonpolar
518	A/G	N/S	173	NA	LRR5 (VS)[1]	Polar/Polar
1519	A/G	I/V	507	NA	LRR18 (HCS)[2]	Nonpolar/ Nonpolar
1556	G/A	Q/R	519	NA	LRR18 (VS)	Polar/Polar
1677	T/C	Y	559	NA	Potential LRRCT	Polar/Polar
1986	G/T	P	662	TIR	TIR	Nonpolar/ Nonpolar
2031	T/C	N	677	TIR	TIR	Polar/Polar
2040	T/C	A	680	TIR	TIR	Nonpolar/ Nonpolar
2053	G/C	L/V	685	TIR	TIR	Nonpolar/ Nonpolar
2091	G/A	K	697	TIR	TIR	Polar/Polar
2127	C/T	S	709	TIR	TIR	Polar/Polar
2138	A/C	H/P	713	TIR	TIR	Polar/ Nonpolar
2220	A/C	P	740	TIR	TIR	Nonpolar/ Nonpolar

[1] Variable segment; [2] Highly conserved segment; NA: not available

In this study, Egyptian buffalo *TLR6* complete coding region (2379 bp) was determined and submitted to GenBank: KM879444. Sequence analysis of Egyptian buffalo *TLR6* coding region depicted, for the first time, the presence of 13 nucleotide polymorphic sites, 69% (n=9) were transitions and 31% (n=4) were transversions. The average polymorphic density was 1 SNP per 183bp. Amino acids translation of the coding sequence resulted in the presence of 6 non-synonymous variants (nsSNP) and 7 synonymous ones (sSNP). The 6 nsSNPs were *I15V*, *N173S*, *I507V*, *Q519R*, *L685V* and *H713P*. They scored 0.70, 0.61, 1.00, 0.51, 0.68 and 0.00 respectively using SIFT. The first 5 nsSNPs were found to be tolerated while *H713P* was found to be not tolerated (<0.05). The latter was also found to change polarity from polar Histidine to nonpolar Proline (Table 2). Polymorphism in *TLR*s has been found to have impact on the genes and sometimes causing diseases. *TLR*s polymorphism was found to be related to uterine diseases such as Endometritis [28] and mastitis [29, 30] in cattle. It is also related to infectious bovine keratoconjunctivitis in the American Angus breed [31] and a

decreased response to paratuberculosis in cattle [32] and in sheep [33]. Pig disease resistance was related to variation in relation to *TLR1*, *TLR2*, *TLR4*, *TLR5*, and *TLR6* [7]. In chicken, *TLR4* is linked to susceptibility to Salmonella [34]. In humans, polymorphism in *TLR* genes were related to endotoxin hyporesponsiveness [35], malaria [36], prostate cancer [37], *TLR4* in gastric cancer [38] and esophageal cancer [39], pelvic inflammatory diseases [40]. Polymorphism in *TLR5* resulting of the stop codon in the LRR increased the susceptibility of human to pneumonia due to the inability to recognize the flagellated bacterium causing it [41]. Whereas a nucleotide variation (359 T>C) in *TLR6* elevated the risk level of Legionnaire disease [42].

TLR6 Egyptian Buffalo coding region sequence was compared with other Indian river buffalo breeds: Murrah buffalo (GenBank: KC153655.1) and Nili-Ravi Buffalo (GenBank: HQ327992.1) [17]. The nucleotide and amino acids similarities in Murrah buffalo were 99.9% (2405/2408) and 100% (with one amino acid difference) and in Nili-Ravi buffalo 97.1% (2316/2385) and 95.46%, respectively. Similarity of *TLR*6 Egyptian Buffalo sequences with *Bos taurus* [GenBank: NM_001001159], *Ovis aries* [GenBank: NM_001135927] and *Capra hircus* [GenBank: NM_001285540.1] were 98.6%, 96% and 94.4% for nucleotides and 97.9%, 93.8% and 90.7% for protein, respectively. Interestingly, nucleotide differences reported in Nili-Ravi buffalo compared to Egyptian buffalo included an insertion of three successive nucleotides that affected two amino acids. This insertion was present in sheep and goat but not in *Bos taurus*. This finding has led Dubey *et al*. [17] to relate buffalo to sheep than to cattle. However, the close relation between cattle and buffalo has been well established being from subfamily Bovinae, whereas sheep and goat are from subfamily Caprinae.

Signal peptide of *TLR6* in Egyptian buffalo breed was absent, as determined by SignalP 4.1 [22], being a non-secretory protein as in mice, human and cattle [6]. Using SMART and LRRfinder, the main protein domain architecture of Egyptian buffalo *TLR6* gene comprised of 2 transmembranes, LRR domains, LRRCT, TIR domain. Five LRRs were predicted by SMART; one LRR located at 75-98 (towards the N-terminal) and 4 LRRs: 376-399, 402-425, 450-471 and 472-496 (towards the C-terminal). In addition to two Pfam-LRR: Pfam-LRR_7 (100-116) overlapped by Pfam-LRR_1 (101-121) and Pfam-LRR_7 at amino acids 122-138 located towards the N-terminal. On the other hand, 18 LRRs were predicted by LRRfinder. SMART and LRRfinder predictions complement each other. SMART identify N-terminal LRR (LRRNT) and C-terminal LRR (LRRCT) which in some cases are not identified by LRRfinder whereas LRRfinder can predict other LRRs not identified by SMART [43].

The number of LRR domains (predicted by SMART) of the Egyptian buffalo (5LRRs + 2Pfam-LRR) and their distribution [1 LRR + 2 Pfam-LRR towards the N-terminal and 4 LRRs at the C-terminal] were similar to Murrah buffalo breed. However, Nili-Ravi buffalo breed was different. It had 2 LRRs + 1 Pfam-LRR at the N-terminal and 3 at the C-terminal [17] (Fig.1). Comparing Egyptian buffalo *TLR6* architecture domain with other members of the Bovidae family, it was found to be similar to *Bos taurus* [6] having 1 LRR + 2 Pfam-LRR towards N-terminal and 4 LRR towards C-terminal. However, it differed from goat and sheep. *Capra hircus* has 2 LRR towards N-terminal and 2 towards C-terminal while *Ovis aries* has 2 LRRs towards N-terminal and 4 towards C-terminal (Fig.2).

As mentioned earlier, 13 polymorphic sites were reported in Egyptian buffalo. Six polymorphic sites were involved in amino acid variations: 3 in LRRs, 1 in transmembrane, and 2 in TIR domains (Table 2). Eighteen LRRs were found in Egyptian buffalo using the LRRfinder program. The three amino acid variants were found in LRR5 and LRR18. Since each LRR is composed of variable and highly conserved segments, two amino acid variants were in LRR variable segments; *N173S* in LRR5 and *Q519R* in LRR18. The third amino acid variant *I507V* was in LRR highly conserved segment in LRR18. LRR domain is responsible for the recognition of ligands and subsequently the signaling pathway [11]. Polymorphisms that occur in LRRs may cause changes in responsiveness [13] towards a foreign pathogenic or non-pathogenic microorganism. The amino acid variant *I15V* present in transmembrane domain plays a main role in *TLR* ligand recognition and signal transduction [14]. One amino acid variant *H713P* present in the TIR domain changed polarity from polar Histidine to nonpolar proline and was found to be not tolerated. Changes in polarity can affect the protein function [44] and may affect the TIR role in signal transduction that affects the inflammatory response and the response to invading pathogens [11]. Change in protein function related to change of amino acids polarity of polymorphic site was reported in cattle *TLR4* where the polymorphic site was at the border of TM and TIR regions [45]. The reported genetic variations in river buffalo *TLR6* is expected to affect the resistance or susceptibility to various bacterial infections.

ISSN: 0975-8585

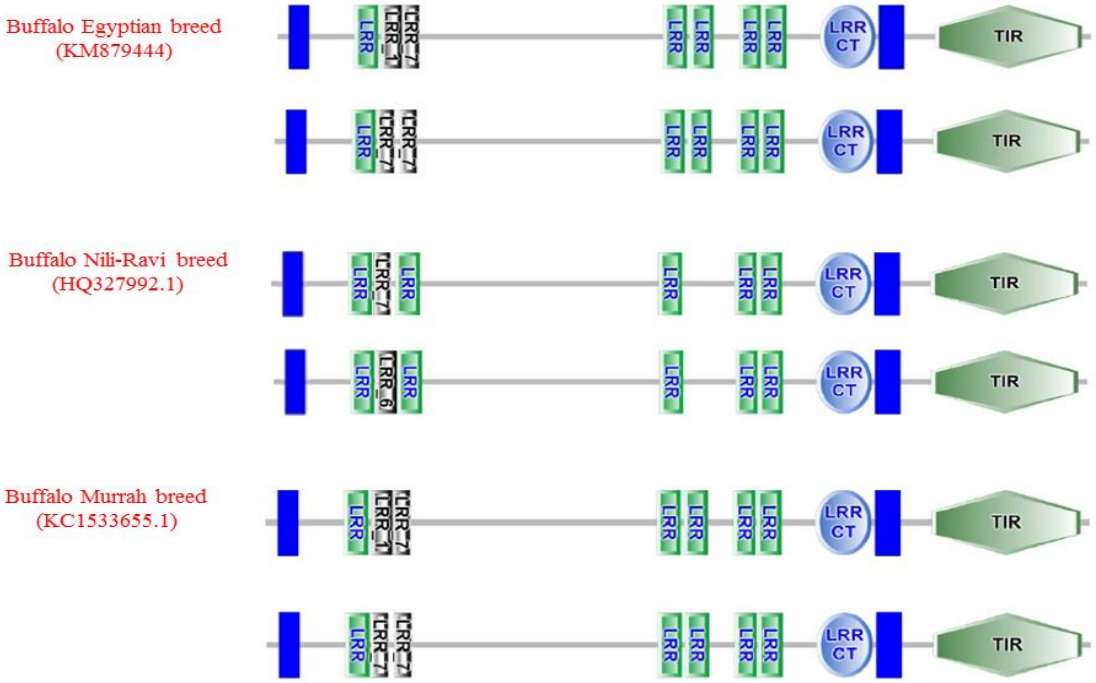

Figure 1: *TLR6* protein comparison in different buffalo breeds.

Egyptian and Murrah buffalo breeds have 1 LRR + 2 Pfam-LRR towards N-terminal and 4 LRR towards C-terminal. Nili-Ravi breed have 2 LRR + 1 Pfam-LRR towards N-terminal and 3 LRR towards C-terminal. The first Transmembrane (Vertical blue line) given in the results by SMART was manually added in the figure since it was not presented in the illustrated SMART graph.

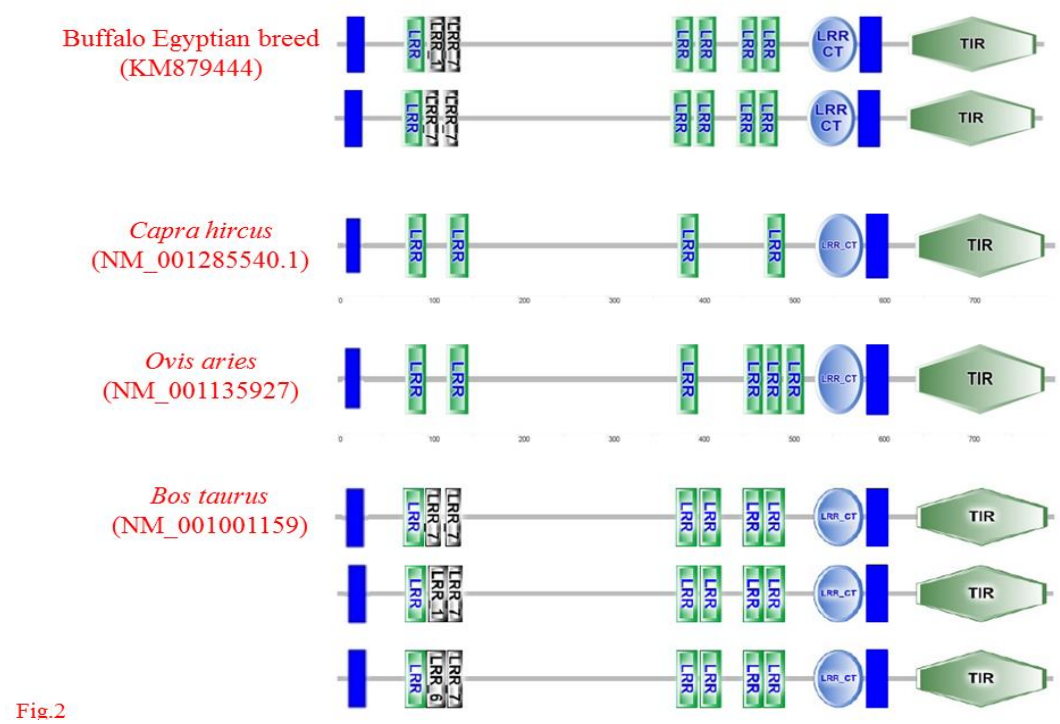

Figure 2: *TLR6* protein comparison between Egyptian buffalo, *Capra hircus*, *Ovis aries* and *Bos taurus*.

Egyptian buffalo breed and *Bos taurus* have 1 LRR + 2 Pfam-LRR towards N-terminal and 4 LRR towards C-terminal. *Capra hircus* has 2 LRR towards N-terminal and 2 towards C-terminal while *Ovis aries* has 2 towards N-terminal and 4 towards C-terminal. The first Transmembrane (Vertical blue line) given in the results by SMART was manually added in the figure since it was not presented in the illustrated SMART graph.

ISSN: 0975-8585

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