

## Single Nucleotide Polymorphisms in *TLR4* Gene and Endometritis Resistance in River Buffalo (*Bubalus bubalis*)

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### Abstract

Toll-like receptor (TLR) genes play a crucial role in pathogen recognition and subsequent activation of the innate immune response. The role of *TLR4* in the initiation of inflammatory and immune response makes it a suitable candidate gene for marker-assisted selection to enhance disease resistance in dairy animals. The present study was undertaken to characterize the distribution of single nucleotide polymorphisms (SNPs) in *TLR4* coding region, and to test their role as potential risk factor for the occurrence of endometritis in river buffalo Egyptian breed (*Bubalus bubalis*). The analysis of the entire coding sequences (CDs) enabled the identification of 13 non-synonymous SNP. A statistical correlation was observed between resistance to endometritis and the genotype CC at the 2464 C > A locus and AA genotype at the 2465 A > C. Moreover, a correlation was seen between susceptibility to endometritis and the heterozygous and recessive homozygous genotypes of these SNPs. Haplotype reconstruction of the *TLR4* gene revealed a statistical significance of haplotype frequencies between healthy and diseased cases by performing a permutation test which retrieved significant association with the occurrence of the disease in the coding region of *TLR4*. PolyPhen 2 analysis revealed two amino acid substitutions which may have a potential structural and functional significance. The current study describes some novel SNPs in *TLR4* gene of river buffalo Egyptian breed and their association with the endometritis disease, therefore suggesting a possible positional marker in the buffalo genome that may be related to such a disease.

**Keywords:** *Bubalus bubalis*, *TLR4*, Endometritis, SNPs, Genotype.

### 1. Introduction

Water buffalo plays a pivotal role in the livestock and agricultural economy of many developing countries including Egypt. The current world water buffalo population is estimated at 195 million heads including both river and swamp buffaloes. Egyptian buffaloes are of the river type with a population of 4 million heads, it ranks third after the Indian (over 115 millions) and Pakistani buffalo (31.7 millions) (Lewandowski 2015).

Although there are many advantages for raising water buffalo, breeders and farmers have been facing many problems such as poor reproductive efficiency and high incidence of infertility (Michelizzi *et al.*, 2010). Endometritis is the most common uterine disease observed in buffaloes slaughtered at abattoirs, and is one of the main causes of infertility in both cattle and buffalo (Ajevar *et al.*, 2014; Azawi *et al.*, 2008). The severity of endometritis is dependent in part on the type of bacteria present; moreover, the establishment and persistence of the uterine infection are also influenced by the presence of a suitable

uterine environment, genetic factors, and the animal's innate and acquired immunity (Williams *et al.*, 2005).

Mammalian Toll-like receptors (TLRs) are a key family of innate immune proteins and a major class of pattern-recognition receptors (PRRs) (Medzhitov 2001). TLRs recognize broad classes of pathogen-associated molecular patterns (PAMPs), and play an essential role in initiating and directing immune responses to pathogens (Takeda *et al.*, 2003; Takeda and Akira, 2005).

Single nucleotide polymorphisms (SNPs) can occur anywhere in the genome. Leveque *et al.*, (2003) reported that polymorphisms within TLR genes are associated with variations in disease resistance traits in livestock.

The *TLR4* gene in water buffalo consists of three exons and two introns, and is located on chromosome number 8 (Gulhane and Sangwan, 2012). *TLR4* is a member of the TLR family which is highly expressed in the endometrial cells of the uterus, and play a key link between endometritis and immune system response; therefore, they can be considered as detection markers for the endometritis disease (Ganesan *et al.*, 2013; Yeo *et al.*, 2013; Ju *et al.*, 2014). Polymorphisms of *TLR4* exons in buffalo

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lead to the alteration of resistance and susceptibility for the disease occurrence, and make it a suitable candidate gene for use in marker-assisted selection (Mitra *et al.*, 2012).

The aim of this study is to identify SNPs in the full CDs of *TLR4* gene performing a case-control study to test its role as potential risk factor for the endometritis occurrence in river buffalo Egyptian breed, with the purpose of enhancing available genetic tools for the improvement of health and production of an animal considered of increasing worldwide economic importance. Additionally, the current study characterizes *TLR4* protein architecture domains, where TIR domain plays an important role in inflammatory response activation since the number and structure of LRRs of TLRs have a significant effect on the function of pathogen recognition system among species.

## 2. Materials and Methods

### 2.1. Study Design

Genetic association between *TLR4* gene SNPs and endometritis disease was studied by a case-control approach on uterine tissues which were collected from forty Egyptian buffaloes (river buffalo) at the slaughter house. The forty samples were divided equally to apparently healthy uteri and clinically infected ones according to the physical examination and presence of abnormal secretions and inflammation signs in uterine tissues (Williams *et al.*, 2005; Sheldon *et al.*, 2009). Bacteriological processing was performed on all forty uterine samples to confirm the samples identification detected by visual inspection, and to identify bacterial pathogens from clinically diseased samples (Collee *et al.*, 1996; Quinn *et al.*, 2011).

### 2.2. *TLR4* Sequencing

Total genomic DNA was extracted from uterine samples using DNeasy Blood and Tissue kit (Qiagen, USA) according to the manufacturer's instructions. Five specific PCR primers were designed to amplify entire CDs of three exons of *TLR4* gene using published *Bubalus bubalis**TLR4* gene (accession no. JN786600) and the web interface primer3 as shown in Table 1. The *TLR4* coding sequence was amplified using five primers according to the following thermal profile: initial denaturation at 95°C for 1-3 minutes, followed by forty cycles of thirty seconds at 95°C, thirty seconds at  $T_{ann}(C)$  which is the annealing temperature specific for each primer (Table 1) and one minute at 72°C, with a final extension for one minute. PCR reactions were performed with PCR Master Mix kit (Thermo Fisher Scientific, UK), which included in addition, 50ng of genomic DNA and 10µM of each primer (forward and reverse) in a final volume 50 µL. Amplicons were purified according to the manufacturer's instructions (QIAquick PCR Purification Kit, Qiagen) and were bi-directionally sequenced by Laragen company (USA).

**Table 1.** PCR primers, annealing temperatures and amplicon size of amplified *TLR4* exons.

Amplicon (bp)	PCR $T_{ann}(C)$	Sequence (5'-3')	CDs of <i>TLR4</i> gene
785	53°C	F: GACAGCCATCTATAAGCCAAGG R: TGCTGTGTTGCAAATGAACCT	TLR4 A (CDs_Exon1)
273	56°C	F: AGAGTTGCTGGGAAGTCTGC R: AACATTCCTCCTTGACAGTGGT	TLR4 B (CDs_Exon2)
985	55°C	F: GCATTGTTATATCTGTGTGGAGACC R: TGAGATCTAGATACTGAAGGCTTGG	TLR4 C (CDs_Exon3)
953	54°C	F: CGAATTCTCAGGGGACGATA R: GCTCTGCACACATCATTGTC	TLR4 D (CDs_Exon3)
953	55°C	F: GACTGCAGTTCAACCGTATCA R: TGGCAGCATTACTTGTAACTGA	TLR4 E (CDs_Exon3)

### 2.3. SNP Selection and Genotyping

Sequences were analyzed by multiple alignment using CLUSTAL-W program (Thompson *et al.*, 1994). The forty river buffalo Egyptian breed sequences of the *TLR4* gene were aligned with CDs of publicly available GenBank: JN 786600. Each forward and reverse sequence from a single DNA sample was aligned against each other to generate a consensus sequence for each sample and to identify polymorphisms among the samples, in addition to the confirmation of polymorphic sites by visual examination of sequence's charts.

### 2.4. Genetic Association and Protein Analysis

Genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium (HWE) by using Fisher's Exact test, where the allelic and genotypic frequencies were carried out for each SNP independently.

The univariate logistic regression model was performed for the SNPs that were significantly associated with the disease. Odds Ratio test (OR) was calculated with 95 % confidence interval (CI) (McHugh, 2009). All statistical analyses were performed using R statistical program, and *P*-value was corrected using Bonferroni method (Bland and Altman, 1995). Haplotype reconstruction was performed using PHASE software, (version 2.1) (Stephens *et al.*, 2001). The potential impact on protein structure and function due to amino acid substitutions was performed by PolyPhen-2 software (Polymorphism Phenotyping V2) with default parameters (Adzhubei *et al.*, 2010), and the colored figures are available at <http://genetics.bwh.harvard.edu/cgi-bin/ggi/ggi2.cgi>. Protein domain has been predicted from the CD sequence by using the Conserved Domain Database available at (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) (Aron *et al.*, 2009).

## 3. Results and Discussion

### 3.1. Bacteriological Analysis

In a previous study, Osman *et al.* (2015) aimed to characterize the transcripts of TLRs: TLR1, TLR2, TLR4, TLR6 and TLR10 in *Bubalus bubalis* with endometritis. Quantitative real-time (q-PCR) assays were performed to detect transcript expression profiles of these TLRs in liver, mammary gland, ovary and uterus of *Bubalus bubalis* with and without endometritis. The results showed that the

transcript profiles of TLRs especially TLR2 and TLR4 were significantly different in mammary glands, ovary and the uterus.

The current study focused on an important *TLR* gene, *TLR4*, which has the ability to recognize endotoxins associated with Gram-negative bacterial infections (White *et al.*, 2003 ; Davies *et al.*, 2008; Mariotti *et al.*, 2009 ; Mitra *et al.*, 2012 ; Alfano *et al.*, 2014). As shown in Table 2, *Escherichia coli* was the highly isolated bacterium from the diseased samples.

**Table 2.** Identification of bacteria, isolated by aerobic and anaerobic culture of uterine swabs, according to their expected pathogenic potential in the uterus.

1	2	3
<i>E. coli</i> (45.5)	<i>S. aureus</i> (31.58)	<i>Micrococcus spp.</i> (23.68)
	<i>S. pyogenes</i> (26.32)	<i>Klebsiella spp.</i> (33.3)
		<i>S. epidermidis</i> (18.42)
		<i>Proteus spp.</i> (21.2)

-Categories are: (1) recognized uterine pathogens associated with uterine endometrial lesions; (2) potential pathogens frequently isolated from the bovine uterine lumen and cases of endometritis but not commonly associated with uterine lesions; (3) opportunist contaminants transiently isolated from the uterine lumen and not associated with endometritis. Numbers in parenthesis indicate the percentage of a total of 20 samples isolates.

### 3.2. SNPs Identification and Protein Analysis

The analysis of the entire CDs of *TLR4*, which was deduced from five overlapped amplified fragments, enabled us to identify thirteen polymorphic sites. All SNPs were bi-allelic and non-synonymous with eight nSNPs being transversions and five nSNPs being transitions. In addition, two dinucleotide SNPs in the CDs of exon 3 (576/577 TG > GA & 2464/2465 AC > CA) were identified. The *TLR4* CD sequences generated from healthy and diseased cases were submitted to GenBank with the accession numbers KU984440 and KU984441, respectively. For protein analysis, the majority of amino acid substitutions were detected to be benign. Only two substitutions (I 271 R and F272 L), which were found within *TLR4* coding region, were observed to be possibly damaging, and may have a potential impact on the alteration of protein structure and function (Adzhubei *et al.*, 2013). It is to be noted that the change of polarity from nonpolar Isoleucine to polar Arginine (I 271 R) might also affect the protein structure and function (Allen *et al.*, 2013). This effect of change in protein function, related to that of amino acid polarity of *TLR4* SNPs, was also reported in cattle by White *et al.* (2003). It was also previously reported that non-synonymous SNPs that modify amino acids polarity in TLRs may affect ligand binding and recognition (Zhang *et al.*, 2014). Furthermore, amino acid substitutions that alter the amino acid polarity may also have a potential impact on host immune responses and resistance to diseases (Shinkai *et al.*, 2006).

Table 3 shows all the SNPs, their positions in *TLR4* CDs, the protein domain and PolyPhen-2 analysis of amino acid substitution effects of these SNPs. Five non-synonymous SNPs were detected in Leucine-rich repeats (LRR) domains of *TLR4*, which might have a role in altering its ability to identify extracellular pathogens

(Fujita *et al.*, 2003 ; Seabury *et al.*, 2007). Furthermore, polymorphisms that occur in LRR domains may cause changes in responsiveness towards pathogenic microorganisms (Matsushima *et al.*, 2007).

### 3.3. Case-Control Study Analysis

As a first step for the genetic association study, Fisher's Exact test was carried out for the calculation of allele and genotype frequencies for each SNP (McDonald, 2009), where it was found that all *TLR4* SNPs conformed to Hardy-Weinberg equilibrium ( $P > 0.05$ ) (Salanti *et al.*, 2005). Only two SNPs (2464 M (C/A) & 2465 M (A/C)) in the coding region were found to be statistically significant ( $P < 0.01$ ) in the distribution of allele and genotype frequencies between healthy and diseased cases (Table 4).

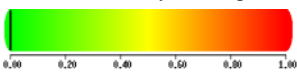
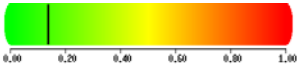
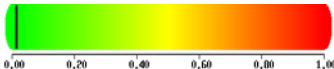
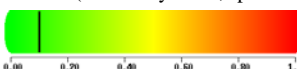
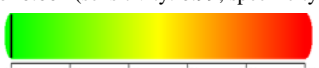


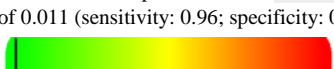
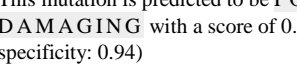
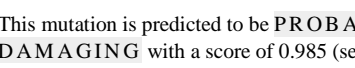
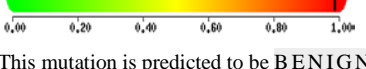
### 3.4. Odd Ratio Test Analysis for Statistically Significant SNPs

The two statistically significant SNPs [2464 (C/A) and 2465 (A/C)], associated with endometritis disease ( $P < 0.01$ ) (Table 4) were subjected to further analysis. The logistic regression model was performed to calculate the Odd Ratio (OR) for each SNP.

The OR test analysis revealed the presence of associations between the two statistically significant SNPs [2464 (C/A) and 2465 (A/C)] and resistance/susceptibility to the endometritis disease. Specifically, a correlation was observed between resistance to the endometritis disease and the genotype CC at the 2464 C > A locus and AA genotype at the 2465 A > C locus [ $P = 0.006$ , OR = 0.2411 and 95% CI = 0.134-0.436]. Moreover, a correlation was detected between susceptibility to endometritis and the heterozygous and recessive homozygous genotypes of those SNPs (OR: 2.125 and 6.00;  $P$ : 0.01 and 0.002, respectively; Table 5). Despite the relatively small number of samples used, the results give a rather good idea about the association between *TLR4* genotypes and resistance/susceptibility to endometritis. Those results are in good agreement with: Cargill and Womack, 2007 ; Uenishi and Shinkai, 2009 ; Jann *et al.*, 2009 ; Koets *et al.*, 2010 ; Plantinga *et al.*, 2012 ; Tschirren *et al.*, 2013 ; Alfano *et al.*, 2014, who reported that TLRs polymorphisms are associated with resistance and susceptibility to diseases in many species.

Previous studies have demonstrated the influence of polymorphisms on susceptibility to several bacterial diseases, where *TLR4* polymorphisms were found to increase the risk of infections like tuberculosis (Zhang *et al.*, 2013 ; Sun *et al.*, 2015), urinary tract infections (Hawn *et al.*, 2009), Mastitis (Kannaki *et al.*, 2011; Sharma *et al.*, 2006), and other disease conditions (Mucha *et al.*, 2009 ; Schnetzke *et al.*, 2015). The relation between endometritis occurrence and genetic polymorphism of TLRs in humans and cattle was previously reported ( Taylor *et al.*, 2012 ; Pinedo *et al.*, 2013). In this respect, several polymorphisms have been found in the bovine *TLR4* gene, 12 SNPs affecting ligand binding domain (White *et al.*, 2003), and four SNPs were associated with susceptibility to Map infection (Mucha *et al.*, 2009).

**Table 3.** Detected SNPs and their effect on protein function in *TLR4* CDs.

CDs_Exon	SNPs positions	Amino acid substitution	Protein domain	Amino acid substitution effect PolyPhen-2 software (Polymorphism Phenotyping V2) [Adzhubei <i>et al.</i> 2010]
CDs_Exon1	44 Y(C>T)	Thr (Polar) 15 Met (Non Polar)	-*	This mutation is predicted to be <b>BENIGN</b> with a score of 0.000 (sensitivity: 1.00; specificity: 0.00) 
CDs_Exon 1	66 W(A>T)	Arg (Polar) 22Ser (Polar)	LRR_R1	This mutation is predicted to be <b>BENIGN</b> with a score of 0.137 (sensitivity: 0.92; specificity: 0.86) 
CDs_Exon 3	572 M (C>A)	Tyr (Polar) 191Ser (Polar)	LRR_8	This mutation is predicted to be <b>BENIGN</b> with a score of 0.013 (sensitivity: 0.96; specificity: 0.78) 
CDs_Exon 3	576 K (T>G)	His (Polar) 192Gln (Polar)	LRR_8	This mutation is predicted to be <b>BENIGN</b> with a score of 0.101 (sensitivity: 0.93; specificity: 0.85) 
CDs_Exon 3	577/579 R(GA>AG)	Lys (Polar) 193 Glu (Polar)	LRR_8	This mutation is predicted to be <b>BENIGN</b> with a score of 0.001 (sensitivity: 0.99; specificity: 0.15) 
CDs_Exon 3	647 R(G>A)	Gly(Non Polar) 216 Asp (Polar)	LRR_R1	This mutation is predicted to be <b>BENIGN</b> with a score of 0.055 (sensitivity: 0.94; specificity: 0.84) 
CDs_Exon 3	662 R(G>A)	Gly(Non Polar) 221 Asp (Polar)	-*	This mutation is predicted to be <b>BENIGN</b> with a score of 0.389 (sensitivity: 0.90; specificity: 0.89) 
CDs_Exon 3	672 M(A>C)	Lys (Polar) 224 Asn (Polar)	-*	This mutation is predicted to be <b>BENIGN</b> with a score of 0.011 (sensitivity: 0.96; specificity: 0.78) 
CDs_Exon 3	812 K(T>G)	Ile (Non Polar) 271Arg (Polar)	-*	This mutation is predicted to be <b>POSSIBLY DAMAGING</b> with a score of 0.964 (sensitivity: 0.76; specificity: 0.94) 
CDs_Exon 3	816 M(C>A)	Phe (Non Polar) 272Leu (Non Polar)	-*	This mutation is predicted to be <b>PROBABLY DAMAGING</b> with a score of 0.985 (sensitivity: 0.74; specificity: 0.96) 
CDs_Exon 3	2464/2465 M (AC>CA)	Gln (Polar) 822Thr (Polar)	-*	This mutation is predicted to be <b>BENIGN</b> with a score of 0.001 (sensitivity: 0.99; specificity: 0.15) 

-SNPs positions were calculated by taking the ATG start codon as position 1 on the GenBank sequence: JN786600

- Amino acids substitutions are considered according to the ATG start codon

-\* Protein position without known function

**Table 4.** Corrected P-values for allelic and genotypic frequencies for each SNP of *TLR4* CDS.

SNPs position	Corrected P value of allelic frequency	Corrected P value of genotypic frequency
44 Y(C/T)	0.05	0.14
66 W(A/T)	0.05	0.14
572 M(C/A)	0.97	0.99
576 K(T/G)	0.96	0.98
577 R(A/G)	0.97	0.99
579 R(G/A)	0.97	0.99
647 R(G/A)	0.96	0.98
662 R(G/A)	0.96	0.98
672 M(A/C)	0.96	0.98
812 K(T/G)	0.96	0.98
816 M(C/A)	0.96	0.98
2464 M(C/A)	0.004*	0.0012*
2465 M(A/C)	0.004*	0.0012*

-\*Statistically significant values ( $P < 0.01$ ). Fisher Exact test was used because of the small number of samples. Bonferroni was used for p value correction.

**Table 5.** Polymorphic sites including genotypes of statistically significant differences in frequency distribution between healthy and diseased cases.

SNPs	Genotype	P-value	OR	95% CI
CD 2464	CC	0.006*	0.2411	0.134-0.436
	M(C/A)	0.01*	2.125	1.1962-3.7750
CD 2465	AA	0.002*	6.000	1.969-18.275
	M(A/C)	0.01*	2.125	1.1962-3.7750
	CC	0.002*	6.000	1.969-18.275

-\*Statistically significant p values ( $p < 0.05$ ) and ORs. CL: confidence level.

### 3.5. Haplotype Prediction

The determination of haplotype reconstruction for several SNPs in one gene is considered to be a powerful tool to provide more information about genotype-phenotype associations than individual SNPs (Ciampolini *et al.*, 2007). Haplotype reconstruction based on *TLR4* polymorphisms performed by PHASE software, generated 21 possible haplotypes. The most frequent haplotype within healthy cases was TTCTAGGGATCCA which displayed 30 % frequency, while CACTAGGGATCAC was the most frequent haplotype within diseased cases with a 31 % frequency. The software PHASE revealed the presence of a statistical significance ( $P$  value=0.02) of haplotype frequencies between healthy and diseased cases, which indicated significant association between haplotype frequencies and the occurrence of endometritis disease in the coding region of *TLR4*.

Haplotype reconstruction of TLRs and its association with the disease occurrence have been reported in humans (Ferwerda *et al.*, 2007 ; Bochud *et al.*, 2008), cattle (Jann *et al.*, 2009) and buffaloes (Alfano *et al.*, 2014).

## 4. Conclusion

Polymorphisms and mutations of TLRs interfere with the innate immune activation due to the reduction of the protein ability to recognize pathogen associated molecular patterns (PAMPs). The current study is focused on an important Toll-Like receptor gene, *TLR4*, which has the ability to recognize endotoxins associated with gram-negative bacterial infections. It suggests the presence of novel polymorphic sites in *TLR4* gene in the river buffalo Egyptian breed and their association with the endometritis disease occurrence. Studies of the genetic factors involved in complex diseases may help identify their underlying physio-pathological pathways, which will improve our ability to understand the disease in its entirety, and to determine the risk of developing it. This will pave the way to the improvement of disease resistance in herds by selective breeding, and also to the identification and synthesis of innovative drugs.

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