



The Role of Toll-Like Receptor 4 Gene Polymorphism *TLR4* (Thr399Ile) and Expression in Adult Urinary Tract Infections Pathogenesis

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Abstract: The first line of protection against microbial illnesses is the immune response. Toll-like receptors (*TLRs*) are essential for innate immunity, and toll-like receptor 4 (*TLR4*) is recognized as a key pattern-recognition receptor (PRR) for identifying gram-negative bacteria's lipopolysaccharide (LPS). Numerous *TLR* genes have been shown to have single nucleotide polymorphisms (SNPs), which may be connected to the susceptibility or resistance to specific infections and other inflammatory diseases. The aim of this study was to determine whether there is a connection between chronic UTI and the *TLR4* (Thr399Ile) gene polymorphism and *TLR4* expression level. A case-control study was conducted on 52 adults. This study uses HRM analysis to look for *TLR4* (Thr399Ile) polymorphisms in UTI patients. *TLR4* expression in the monocytes of UTI patients and healthy controls was found using real-time PCR. *TLR4* (Thr399Ile) genotype and *TLR4* (1196) T allele prevalence were higher in UTI patients than in controls, particularly in cases of acute cystitis and urethritis. Compared to healthy controls, chronic UTI patients had considerably greater levels of *TLR4* expression. The results of the current study, show the presence of a correlation between the genotypes of the *TLR4* gene and the incidence of development of Urinary Tract Infection, as the results showed the significant difference between patients and healthy controls when the genotype Heterozygous (Thr / Ile) with (OR= 0.638), while the genotype Homozygous (Ile / Ile) showed no significant difference between the patients and control group with (OR= 1: Reference). *TLR4* C/T (Toll-like receptor-4 gene polymorphism). The results of gene expression of *TLR4* revealed that this receptor was increased among UTI (3.50 ±0.41 fold) infected Patient. (1.20 ±0.07 fold) in comparison with the healthy control. The findings showed a connection between adult UTI, particularly severe cystitis and urethritis, and the *TLR4* (Thr399Ile) allele carrier status. Chronic UTI and *TLR4* expression levels are linked.

Keywords: Gram-negative sepsis; *TLR4* (Thr399Ile) polymorphism ; gene expression .

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Introduction

Urinary tract infections, sometimes known as UTIs, can affect any region of the urinary tract. The role of *TLR4* in UTIs. One of the most prevalent infections seen in nearby primary care. Adult males under 50 years of age rarely have UTIs, while women are 30 times more likely to get one than men are. Amoxicillin-clavulanate is effective as a first-line antibiotic for

acute community-acquired, uncomplicated UTIs, when *Escherichia coli* is the most common uropathogen. If directed by an adequate history, examinations, and medications to produce positive results and reduce antibiotic resistance, family doctors are capable of managing the majority of UTIs (1,2). Innate immunity is the first line of defense against infection.

Different soluble substances that are produced into urine and anatomical barriers like the glycoprotein plaque uroplakins operate as barriers against microbial colonization (3,4). Further defending against infection are the epithelial cells and several resident immune cells that lining the urine tract. These safeguards prevent bacteria from entering the urinary tract and developing a long-lasting disease (5, 6). The normal flora in the periurethra is thought to be the first line of defense against UTIs and is crucial in the development of resistance to pathogen colonization. If bacteria get past the first barrier, other clearance mechanisms such as regular bladder emptying and urine flow, as well as low pH (optimal pH), urea, the presence of iron and glucose, and high urine osmolality, can be thought of as being inhibitory to bacterial development(7). Additionally, bladder washout is improved by the chemical defenses of epithelia, such as the mucous layer that covers the bladder epithelium and slime that is soluble in urine, which prevent bacterial adhesion (8). Toll like receptors (*TLRs*) are key players in "innate immunity," and toll-like receptor 4 (*TLR4*) is regarded as a key pattern recognition receptor (PRR) for detecting gram-negative bacteria's lipopolysaccharide (LPS) (9,10). In humans, the *TLR4* gene (; 9q33.1), which spans a genomic region of approximately 13.3 kb with three exons, encodes a 224 amino acid protein (11). Researchers have examined the association between single nucleotide polymorphisms (SNP) of *TLRs* and urinary tract infections (12). *TLR4* gene is highly polymorphic, (13). Three primers were used, one for the mutant and another for the wild type, and one common reverse primer.

The study investigated the association between single nucleotide polymorphism in toll-like receptors and the incidence of urinary infection.

Material and methods

The study population consisted of (52) in patients with Urinary Tract Infection and there urine culture showed positive result of Gram negative bacteria. All participants were recruited from Abu Ghraib hospital in Baghdad province, during the period from October 2022 to September 2023.taken Five milliliters of venous blood was taken 2 ml in EDTA tubes for polymorphism which kept at -20 until be used for DNA extraction and 300 microliters in trizole tube for gene expression.

DNA extraction and genotyping of *TLR4* gene

Extraction of Genomic DNA by add bio kit (Cat. No. 10023) Genomic DNA was isolated from blood of patients and healthy group in this study the result of isolated DNA showed good concentration and purity of extracted DNA was measured by Quantus Florometer That purity is ranged to (150-200 ng/ul)., The primer used for HRM genotyping was performed on the (Bioer LineGene 4800 qPCR) using SYBR Green master mix. Genetic variations for Rs4986791 SNP were analyzed using amplification with subsequent high-resolution melting curve (HRM) analysis,. Three primers were used, one for the mutant and another for the wild type, and one common reverse (Table 1) (14). Luna® Universal qPCR Master Mix (NEB, USA) is a high-performance reagent designed for high sensitivity and specificity for HRM PCR detection, this Master Mix consists of the hot-start Taq DNA Polymerase and all the components necessary for real-time PCR, including the SYBR Green I dye, dNTPs mixture, protein stabilizers and enhancers, I dye is a dsDNA-binding fluorescent dye suited for HRM analysis that allows a highly efficient, inhibition-free, PCR amplification. Amplification was carried out in a final volume of 20 µl, containing SYBR Master,10 picomole per reaction

for each of the forward and two reverse primers, and DNA in a fixed concentration of 50 ng per reaction. PCR cycling conditions included an initial Taq Polymerase activation step at 95 C for 3 min followed by 45 cycles of denaturation for 25 s at 95 C and annealing/extension step for 30 s at 60 C to allow and 20 s at 72 C. After PCR amplification, the HRM was carried out over the range of 60–95 rising (14).

RNA extraction and gene expression for Toll-like receptor 4

Using Triquick Reagent (Solarbio,

China), the following procedures were followed in order to extract the total RNA from serum. In accordance with the manufacturer's recommendations, sample preparation was done at room temperature (RT). Reverse transcriptase kit AddScript (addbio, Korea) in this study the result of isolated RNA showed good concentration and purity of extracted RNA(80 -120 ng/ul) was measured by Quantus Fluorometer to convert RNA to cDNA. The cDNA (10-20 ng/ul) conversion detailed in (Table 2).

Table (1): Specific primer for TLR 4 polymorphism.

Primer name	Primer sequence
Common Reverse	TTGAAGCTCAGATCTAAATACTTTAGGCTG
T-FW	CTATGTTCTCAAAGTGATTTTGGGACCAT
C-FW	TGCTGTTCTCAAAGTGATTTTGGGACCAC

Table (2): PCR for cDNA conversion.

Step	°C
Priming	25 for 10 min
Reverse transcription	50 for 60 min
RT inactivation	80 for 5 min
Hold	4 ∞

Following the conversion of RNA into cDNA, Luna® Universal qPCR Master Mix (NEB, USA) is used for real-time PCR reaction and the template this time is cDNA reaction component details mentioned in (Table3) and using

specific primers as a and the SYBR Green PCR Kit. With two primers for house keeping gene *GADPHF* (TGCACCACCAACTGCTTAGC); *GADPHR* (GGCATGGACTGTGGTCATGAG)

Table (3): Reaction components for detection of Toll-like receptor 4.

Master mix components	Volume (µl) for each 1 sample
SYBR- Green Master Mix	10
Forward- primer	1
Reverse- primer	1
Nuclease Free Water	4
cDNA- template	4
Total volume	20

Statistical analysis

SPSS version 16 and Microsoft Office Excel 2007 were used to analyze the data. While nominal variables were shown as a number and a percentage, numerical variables were shown as mean+SD. In cases of normal distribution, a student test was employed to compare the mean difference between

any two groups. distribution. The analysis of relationships between nominal variables used ratio, Chi-square, and/or adjusted Chi-square tests. distribution. Odds The analysis of relationships between nominal variables used ratio, Chi-square, and/or adjusted Chi-square tests. Spearman Rank Correlations were investigated using the

correlation coefficient. When the P-value was less than or equal to 0.05, it was deemed significant (15).

Results and discussion

The DNA was extracted by a commercial genomic DNA purification kit, concentration and purity of the DNA were carried out by using Qubit device. The extracted DNA was analyzed by gel

electrophoresis using (1%) agarose gel at voltage 5 v/cm² for 30 min. The result showed that purity was good and ranged between (1.7- 1.9) and the concentration was acceptable ranging from (150-200 ng/ul), the results of gel electrophoresis showed sharp bands of chromosomal DNA as shown in (Figure 1).

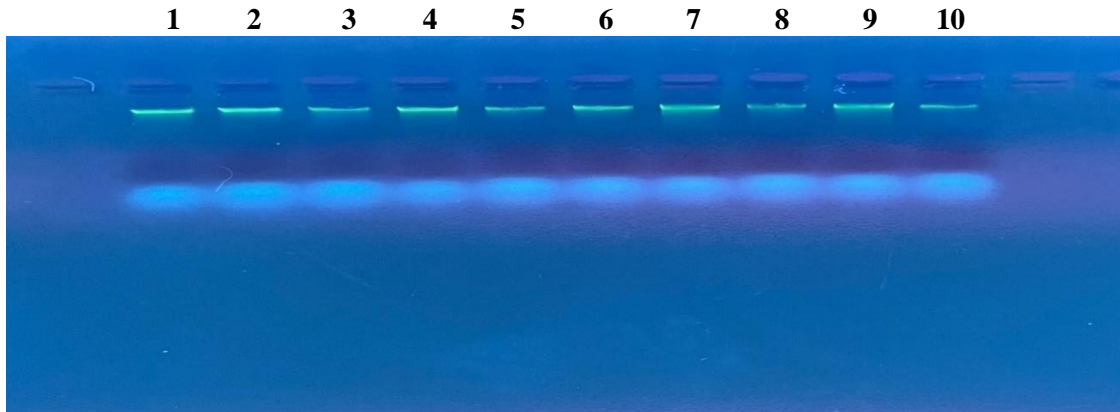


Figure (1): Genomic DNA extraction electrophoresis (1% agarose gel at 75 volt for 30min).

Association between rs4986791 (1196 C > T) and UTI

The innate immune system's Toll-like receptor 4 (*TLR4*), which is only expressed on the bladder and uroepithelial cells and identifies bacterial lipopolysaccharide (LPS), is crucial (17). (also known as 1196C/T corresponding to an Thr399Ile There have been reports of their connection with recurrent UTI (18,19). and replacement mutation). The current study's findings indicate a

relationship between the genotypes of the *TLR4* gene and the likelihood of developing urinary tract infections. The genotype heterozygous (Thr/Ile) results in a significant difference between patients and healthy controls (OR= 0.638), whereas the genotype homozygous (Ile/Ile) results in no significant difference between the patient and control group. (OR= 1: Reference) (Table 4).

Table (4): Genotype and allele frequency of *TLR4* gene in patients and control groups

	Patients No. (%)	Control No. (%)	Chi-Square (χ^2)	P-value	O.R. (C.I.)
TT	23 (71.88%)	16 (80.00%)	1.256 NS	0.262	1: Reference
TC	9 (28.12%)	4 (20.00%)	1.923 NS	0.165	0.638 (0.37-1.24)
CC	0 (0.00%)	0 (0.00%)	0.00 NS	1.00	0.00 (0)
Total	32 (100%)	20 (100%)			
Allele	Frequency				
T	55 (0.86)	36 (0.90)	P-value = 0.0464 *		
Discussion C	9 (0.14)	4 (0.10)	P-value = 0.165 NS		

***TLR4* expression levels in patient infected UTI**

The percentage of *TLR4* expression in healthy controls, chronic UTI patients was 1.20 ±0.07, 3.50

±0.41%, respectively *TLR4* expression in monocytes in patients with chronic UTI was significantly higher than in healthy controls (Table 5).

Table (5): Fold change of *TLR4* gene expression.

Group	No	Fold change
Patients	32	3.50 ±0.41
Control	20	1.20 ±0.07
T-test (P-value)	---	1.194 * (0.0351)
* (P≤0.05).		

Numerous researchers have been intrigued by the connection between the *TLR4* gene's function, expression, and gene mutation and the prevalence of UTI. Arbour was the first to report a link between *TLR4* Thr399Ile polymorphisms and a poor response to the lipopolysaccharide (LPS) of Gram-negative bacteria in mice (20,21). They hypothesized that these *TLR* gene alterations might have an adverse effect on TLR structure or expression, which would then affect how the body responds to bacterial endotoxins. Many more research later verified this (22,23). As a result, TLR4 loses part of its activities as a result of these polymorphisms that change how it interacts with its ligands. According to certain research, the mutation rs4986791 prevents *TLR4* from interacting, which appears to impact the function of *TLR4* expression (reduced to twice the normal level) (24). This decline in functionality is further amplified before *TLR4* and lipopolysaccharide (LPS) form a complex a relationship rs4986791 and gene expression in TLR4 susceptibility to urinary tract infections(13,25,26). Research has revealed how innate immunity affects the likelihood of developing a UTI. Studies revealed that the susceptibility and severity of UTI are influenced by genetic abnormalities or polymorphisms in the innate immune system (16).

Conclusion

The findings showed a connection between adult UTI, particularly severe cystitis and urethritis, and the *TLR4* (Thr399Ile) allele carrier status. Chronic UTI and TLR4 expression levels are linked.

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