Toll-Like Receptor 4 Gene Polymorphisms in patients withUrinary Tract Infection

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Abstract

Background: Urinary tract infection (UTIs) are considered to be the most common infections in humans.
Aims: The present study aimed to investigate the association between single nucleotide polymorphisms (SNPs) in the toll-like receptor 4 (*Tlr4*) gene (*Thr399Ile*) and the incidence of Urinary Tract Infection.
Subjects and Methods:A total of 49 patients with Urinary Tract Infection and 25 apparently healthy control were enrolled in this study. Urine samples from patients with UTI was collected in AL-Imam AL-Hussain teaching hospital in Thi- Qar province, during the period from February 2014 to March 2015.. Urinary isolates were identified by conventional methods.DNA was extracted from the blood samples taken from these participants. *TLR4* gene was amplified with polymerase chain reaction (PCR) using specific primers. Genotyping of the SNPs of interest was done by restriction fragment length polymorphism (RFLP).
Results: The present study was showed 26 (53.06%)isolates *Escherichia coli*; 9 (18.37%) isolates had *Pseudomonas aeruginusa*;7 (14.29 %) isolates had *Klebsiella pneumonia*; 4 (8.16%) had *Proteus* and 3 (6.12%) isolates had *Klebsiellaoxytoca*, the results showed the presence of heterozygous in one sample from study group at site 399 (C / T) after using restriction enzyme *Hinf1*.
Conclusion*: Escherichia coli* was most common causative agent in UTI, *Thr399Ile* may be considered as a risk factor that increases susceptibility to Urinary TractInfection.

Key words: Urinary Tract Infection, toll-like receptor 4, single nucleotide polymorphism

الخلاصة

خلفية الدراسه: يعد التهاب المجاري البوليه من الامراض الشائعه عند الانسان.

الاهداف:هدفت الدراسه الى العلاقه بين التغاير الوراثي في جين TLR-4(*Thr399Ile*) وحدوث الإصابة بالتهاب المجاري البوليه.

الاشخاص وطرق العمل: شملت الدراسة ٤٩ شخصا يعاني من التهاب المجاري البولية وكذلك ٢٥ شخص من الاصحاء اجريت هذه الدراسة في مستشفى الامام الحسين التعليمي في محافظة ذي قار في الفترة من شباط٢٠١٤ الى اذار ٢٠١٥ حيث جمعت عينات البول وشخصت البكتيريا بالطرق المختبرية . عينات الدم جمعت اذ تم استخلاص الحامض النوويواستعملت تقنية ال PCR لمضاعفة جين TLR4 باستخدام بوادئ معينة واستعملت تقنية الRFLP لمعرفة التغاير الجيني لكل من المرضى و الاصحاء.

النتائج:اظهرت الدراسه ٢٦ (٥٣.٠٦ %) عزله *Escherichiacoli*،٩(١٨.٣٧%) 7 (١٤.٢٩%)*Pseudomonasaeruginusa*، *klebsiellapneumonia*، ٤ (٨.١٦%) *proteus* ، ٣(٦.١٢%)*klebsiellaoxytoca*.اظهرت النتائج وجود طفره واحده غير متجانسه لأحد المرضى المصابين بالتهاب المجاري البولية مناصل ٤٩ مصاب في الموقع ٣٩٩ (C / T) بعد استخدام انزيم القطعHinfI .

الاستنتاج :أظهرت نتائج العزلات البكتيرية بان*Escherichia coli*هي العام المسبب الأكثر شيوعا في التهاب المسالك البولية كما واظهرت النتائج الإحصائية للدراسة بان التغاير الوراثي*Thr399Ile* من المحتمل ان يلعب دورا كعامل مساعد للاصابة بالتهاب المجاري البوليه.

Introduction

Urinary tract infections (UTIs) are considered to be most common infections in humans (Bien *et al*.,2012). UTIs are classified into disease categories according to the site of injury: cystitis (the bladder), pyelonephritis (the kidney) and bacteriuria (the urine) (Foxman, 2003). Colonization of the urine in absence of the clinical symptoms is called asymptomatic bacteriuria (ABU) (Bien *et al*.,2012). Most patients with ABU do not need treatment, and in many cases the colonizing by the ABU strains may help to prevent infection by other more virulent bacteria (Hull *et al*.,2000;Darouiche*et al*.,2001;Trautner *et al*.,2003).

In most of the cases UTIs are caused by Gramnegative bacteria from the intestinal flora (Köves, 2014). The primary causative agents responsible for more than 80% of all UTIs including both ABU and symptomatic UTIs, are strains of uropathogenic*E. coli* (Sadler *et al*.,1989;Hooton and Stamm,1997; Svanborg and Godaly,1997).

Toll-like receptors (TLRs) are transmembranous signaling receptors which play a key role in the innate and adaptive immune response, since they are involved in the regulation of inflammatory response and activation of the adaptive immune cellsto reduce infectious pathogens and cancer cells (Iwasaki and Medzhitov,2010). To date, ten different types of TLRs have been described in human which are capable of specifically recognized different pathogens

and/or endogenous damage molecules (Iwasaki and Medzhitov, 2004). TLR4 is one of the most prominent members of TLRs which is present in immune and non- immune cells.

The activation of the innate immune response in the urinary tract is dependent on recognition of bacterial components, products by TLRs (Hedlund*et al*., 2001;Andersen-Nissen*et al*., 2007). In recent years, it has become clear that the immune activation of bladder and kidney epithelial cells depends on TLRs, including TLR4, TLR5, and TLR11 (Samuelsson *et al*.,2004; Song and Abraham, 2008).

TLR4 gene is highly polymorphic, and to date, 15 polymorphisms in its coding sequence have been identified (Schroder and Schumann, 2005). Two common cosegregated single nucleotide polymorphisms (SNP) on the human TLR4 gene were reported. One SNP is an Adenin (A) to Guanin (G) substitution at nucleotid position 896 from the start codon of the TLR4 cDNA. The single nucleotide exchange results in replacement of a conserved aspartic acid residue with glycine at amino acid position 299 (Asp299Gly) (dbSNP databank: rs4986790), The second missense polymorphism results in a change of cytosin (C) to thymin (T) at nucleotid position 1196 from the start codon (dbSNP databank: rs4986791), which causes replacement of a nonconserved threonine with an isoleucine at amino acid position 399 (Thr399Ile) in the extracellular domain of the TLR4 receptor. The average incidence of these polymorphisms is about 10% in the Caucasian population (Reismann, 2009).

The present study was aimed to investigate the association of Thr399Ile SNPs in *Tlr4* gene with incidence of UTI in Iraqi patients.

Subjects and Methods

The study population consisted of 49 (2-75 years old, mean 39.96+16.88, 17 males and 32 females) in patients with Urinary Tract Infection and there urine culture showed positive result of Gram negative bacteria., and 25 age matched (10 males and 15 females) healthy controls. All participants were recruited from AL-Imam AL-Hussain teaching hospital in Thi- Qar province, during the period from February 2014 to March 2015.

Urine samples were taken by standard mid-stream “clean catch” method from patients and Five milliliters of venous blood was taken in EDTA tubes which kept at -20 until be used for

DNA extraction.The urine samples were cultured on plates of Blood agar and MacConkey agar media and the sample plates were incubated at 37°C for overnight. The cultures were subjected to identification of the organisms by using microscopical and macroscopical examinations and routine biochemical tests (Vandepitte*et al*., 2003).

DNA extraction and genotyping of *TLR4* gene

DNA was extracted from the blood samples by using manual method(Sambrook*et al*., 1989). The primer used for amplification of *TLR4* gene (Bioneer/Korea) are shown in table 1.

Template DNA (3µL) from each sample, (1µL) from each primers were added and (10 µL) of Deionized sterile H2O wasadded to each master-mix tube (50 µL PCR master-mix, Bioneer/Korea). The mixture then put in shaker. After mixing, the master-mix tubes were transferred to the thermo cycler (BioRad/Singapore) which is previously programmed with certain protocol according to gene to be amplified.cycling conditions were an initial denaturation for 5 min at 95 ºC, followed by 35 cycles of denaturation at 95 ºC for 40 sec, annealing at 60 ºC for 40 sec, extension at 72 ºC for 50 sec, followed by final extension at 72 ºC for 5 min.

For digestion (10 µL) from *Thr399Ile* PCR products was mixed with a 1µL 10X buffer R and 1µL*Hinf1* (10U) restriction enzyme. Deionized sterile H2O was used to adjust the volume to 30µL. The mixture was then incubated 37C at overnight.

Agrose gel electrophoresis

A 2% gel was prepared, and 10 µL aliquot of digestedPCR product from each sample was loaded into the wells. After 1 hour of electrophoresis , the gel was previously prepared, stained with ethidium bromide (Biobasic/Canada) (0.5 µL/mL). The amplified products were determined by comparison with a commercial 3000 bp ladder (Kappa Biosystem/USA).

Table 1: Specific polymerase chain reaction primers and restriction enzymes for the Thr399Ile.

Gene	*SNP*		***Primer sequences***	*product*	*Enzymes*
TLR-4	*Thr399Ile*	*F* *R*	*5`-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3`* *5-`ACCTGAAGACTGGAGAGTGAGTTAAATGCT-3`*	*406*	*Hinf I*

Statistical analysis

Data were analyzed using SPSS version 16 and Microsoft Office Excel 2007. Numeric variables were presented as mean ±SD while nominal variables were expressed as number and percentage. student test was used to compare mean difference between any two groups in case of normal distribution. Odds Ratio, Chi-square and or corrected Ch-square tests were used for the study of associations between nominal variables. Spearman Rank Correlation coefficient was used to study correlations. P-value was considered significant when it was less than or equal to 0.05.

Results

Types of Bacterial isolates

Patients with *E. coli* infection accounted for 26 (53.06%); other culture results were as follows: 7 (14.29 %) *K. pneumonia*, 9 (18.37%) *P. aeruginusa*, 4 (8.16%) Proteus and 3 (6.12%) *K.oxytoca* . All control subjects were free of infection. These results are shown in table 2.

Table 2: Types of isolated bacteria in patients enrolled in the present study.

Types of isolated bacteria	No.	%
E. coli	26	53.06
K. pneumonea	7	14.29
P.aeruginusa	9	18.37
Proteus	4	8.16
K.oxytoca	3	6.12
Total	49	100.00

Distribution of patients and control subjects according to TLR4 C/T (Toll-like receptor-4 gene polymorphism)

The enzyme *Hinf*I recognizes the sequence GANTC, and accordingly, it cuts PCR product of homozygous mutant genotype (TT) into two bands (377 and 29 bp), while heterozygous genotype (CT) is cut into three bands (406, 377, and 29 bp), whereas, homozygous wild genotype is not affected (the band size is 406 bp) (figure 1).

The results of the current study, show the presence of a correlation between the genotypes of the TLR4 gene and the incidence of development Urinary Tract Infection, as the results show the significant difference between patients and healthy controls when genotype Heterozygous (Thr / Ile) with (OR= 1.577), while the genotype Homozygous (Ile / Ile) show no significant difference between the patients and control group with (OR= 0.525) Table (3) .

Table 3: Distribution of patients and control subjects according to TLR4C/T (Toll-like receptor-4 gene polymorphism).

Genotype	Control	%	Patients	%	OR	95% CI
Wild type	25	100	48	97.9	1.0	……
Homozygous	0	0	0	0	0.525	0.010-27.284
Heterozygous	0	0	1	2	1.577	0.062-40.129

OR: Odd Ratio

CI: Confidence Interval

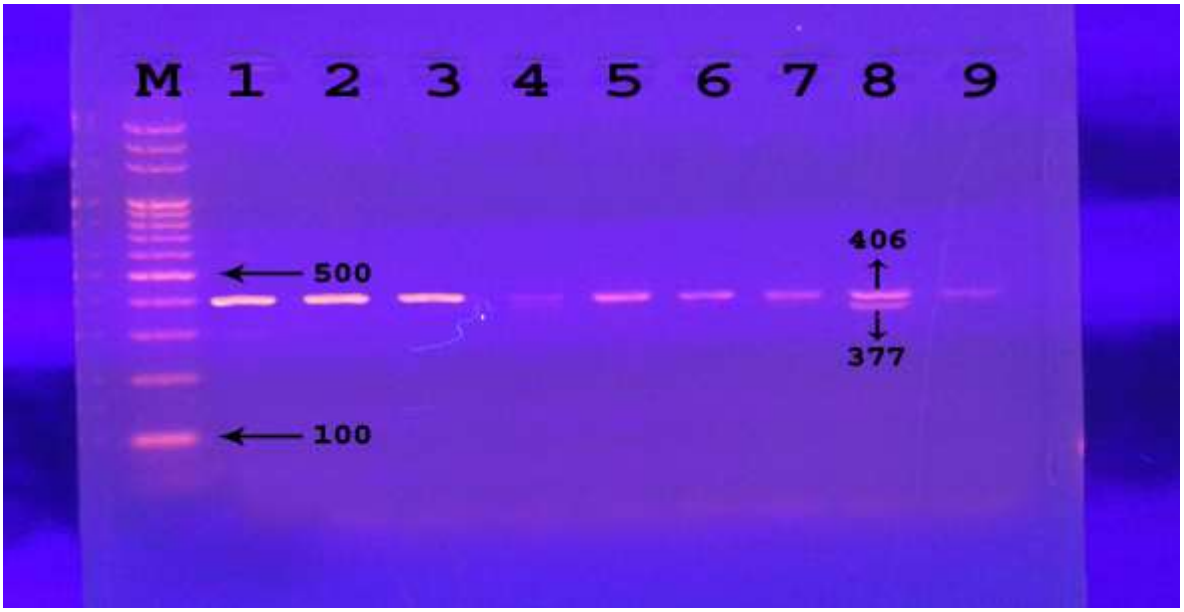

Figure 1 : Agarose gel electrophoresis for digested TLR-4 gene of UTI patients. Bands were fractionated by electrophoresis on a 3% agarose gel (1 h., 80V/cm, 1X Tris-acetic buffer) and visualized under U.V. light after staining with ethidium bromide staining.(M :100 - 3000bp ladder); using restriction enzyme HinfI . Lane: 1, 2,3,4,5,6,7,9 (Intact sample G Allele 406 bp fragment or wild type C/C); Lane:8); (mutant sample C/T or mutant heterozygous) .

Discussion

Bacterial isolates.

Microorganisms isolated from (49) Gram -ve culture-proven bacteriuric are shown in table (2).*E. coli* form the majority of strains isolates 53.06% while *pseudomonas aeruginusa; Klebsiella pneumonia*; *proteus* sp.; Represents 18.37% , 14.29%, 8.16% respectively .these finding are different from that of other works (Nicolle, 1993;AL- Dujaily*et al*.,2003) .*E coli* is an important pathogen in urinary tract, particularly uropathogenic strains through possessing adhesion pili and other adhessins that predispose bacterial binding to the urothelium (Jasmina et al. 2001, Soderhall, 2001). In addition to that *E. coli* possess many other tools make it potent pathogen to urinary tract and other sites of the body (Brooks *et al*., 2007). So for the above mentioned criteria *E. coli* took the first rank of isolation from urinary tract infection in this study.

TLR4 C/T (Toll-like receptor-4 gene polymorphism)

The results of statistical analyses for Toll-like receptor-4 gene polymorphism showed that only one patient (2.04%) had Mutant heterozygous type C/T while all other patients and control subjects showed Wild homozygous type C/C, as shown in table(3). These results indicate that there correlation between polymorphism of TLR-4 gene and Urinary Tract Infection .

This result is not in accordance with that obtained by of Al-Mayah*et al*. (2014) whom Found no significant association of Bladder Cancer with SNPs (Thr399Ile). Yoon. (2006), whom found no genetic polymorphisms were detected in Patients with Bacteremia of this study, suggesting that it is very rare in Korean.

Also Chaloob and Abdul-Mohsen,(2014). Found that The SNPs Thr399Ile may not be considered as a risk factor that increases susceptibility to T. vaginalis infection.

And these findings were agree with the result of Zhu *et al*. (2013) found that the two SNPs Asp299Gly and Thr399Ile were significantly associated with increased risk of overall cancers.

Susceptibility to lethal infections throughout a person's lifetime may be significantly dependent on genetic factors such as genetic polymorphisms (Lin and Albertson, 2004; Angus *etal.*, 2003). The role of a TLR4 polymorphism on the susceptibility to infections is still

controversial and it is currently unresolved whether a hyporesponsive LPS signaling pathway is beneficial or detrimental to the host (Arbour*et al*., 2000; Agnese *et al*., 2002; Morre*et al*., 2003).

TLR-4 gene polymorphism may associated with susceptibility UTIs, but this relationship could vary in different populations and disease types. Further surveys of more cases and different races are needed to make conclusive statements (Yin *et al*., 2010).

A separate study found that both D299G and T399I wereassociated with systemic inflammatory hyporesponsivenesafter LPS inhalation (Michel *et al*., 2003). It has been reported that genetic polymorphisms vary according to race and certain other factors (Nakada*et al*., 2005; Yoon et al.,2006;), In particular, Asian people seem to have a very rare TLR-4 mutation Thr(399)Ile polymorphisms (Nakada.2005; Yoon *et al*., 2006; Hang *et al*.,2004).

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