ASSOCIATION OF *TLR 5* AND *ESCHERICHIA COLI FLIC* POLYMORPHISMS WITH RECURRENT URINARY TRACT INFECTIONS IN WOMEN

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ABSTRACT

This study was aimed to investigate the role of some Toll-like receptor 5 (TLR5) and *E. coli fliC* gene polymorphisms with increased risk to recurrent urinary tract infections (rUTI). From 180 specimens (blood and urine) were collected from women of different age, 60 of them serve as control while 120 had rUTI symptoms. After culturing of urine specimens, 43 (35.8%) were identified as *E. coli* isolates. Four SNPs were identified when amplified and sequenced of *TLR5* include rs5744168, rs775385356, rs2072493 and rs5744174. Twelve flagellar antigen serotypes were obtained from *fliC* sequence of 28 isolates. By using Expasy and Clustal Omega programs, the FliC proteins of H-serotypes arranged in different lengths ranging between 324-634 residues. The N-terminal and C-terminal were conserved region, in contrast, the central region was variable poorly preserved. The results also showed list of conserved amino acids in both FliC termini included L89, Q90,R91, L95, Q98, N101 and E115 in N- terminus in all studied serotypes. Further, the N277 residue that reported its central role for TLR5 interaction were found in some serotypes. Indeed, it was no distinct relation between genotypes or allele frequencies with *fliC* polymorphisms found in *E. coli* isolated from rUTI.

Keywords: stop codon, flagellin, SNPs, 1174 C>T.

ارتباط تعدد الاشكال الوراثية لجين *TLR 5* وجين *fliC* في *Escherichia coli* مع خمج المسالك البولية المتكرر في النساء

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المستخلص

هدفت هذه الدراسة إلى معرفة دور تعدد الاشكال في جين *TLR5* وجين *fliC* في بكتريا *E. coli* مع زيادة خطر الإصابة بخمج المسالك البولية المتكررة. تم جمع 180 عينة (دم وادرار) من نساء بمختلف الأعمار ، ستين منها كانت بمثابة مجموعة سيطرة بينما 120 من نساء لديها أعراض خمج المسالك البولية المتكررة. بعد زراعة عينات البول ، تم تشخيص 43 (35.8٪) من العزلات على انها *E. coli*. تم تحديد أربعة متغايرات (SNPs) عند تضخيمها وتسلسلها لجين *TLR5* شملت كل من rs5744168 و rs775385356 و rs2072493 و rs5744174. تم الحصول على اثني عشر نمط مصلى من المستضدات السوطية من تسلسل *fliC* ل 28 عزلة. باستخدام برامج Expasy و Clustal Omega ، تم ترتيب بروتينات FliC للأنماط المصلية H بأطوال مختلفة تتراوح بين 324–634 من الاحماض الامينية كانت منطقة النهاية الامينية والكاربوكسيلة محفوظة, في المقابل ،المنطقة الوسطى متغايرة بشكل كبير. أظهرت النتائج قائمة بالأحماض الأمينية المحفوظة في كل من ين بروتين FliC المتضمنة L89 و Q90 و R91 و L95 و Q98 و N101 و E115 في الطرف الاميني في جميع الأنماط المصلية المدروسة. تم العثور على N277 التي لها دور مركزي في تفاعل TLR5 مع البروتين في بعض الانماط المصلية في الواقع ، لم تكن هناك علاقة واضحة بين الأنماط الجينية أو ترددات الأليل مع تعدد الأشكال لجين *fliC* الموجود في الإشريكية القولونية المعزولة من خمج المسالك البولية المتكررة.

الكلمات المفتاحية: طفرة التوقف، الفلاجلين, التغايرات الوراثية,1174 C> T.

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INTRODUCTION

Recurrent urinary tract infections (rUTIs)) is described as two incidences of severe bacterial infection of the lower urinary tract with related symptoms in the previous six months or three incidents in the previous year (17). These frequent infections have the same etiology as uncomplicated UTIs. The normal flora of the gut generally contaminates the perineum region and urethra. So bacteria can easily spread from there and infect the bladder (2, 4). Bacteria of the vaginal women play a critical role in the pathogenesis of UTIs, but the normal flora of gut is the main source of bacterial strains responsible for both upper and lower urinary tract infection in the majority of cases, indicating the interconnectivity and interaction of these two habitats (9). Uropathogenic *E.coli* is the leading cause of community-acquired UTIs, accounting for 80–90% of cases (16). UTI can also be caused by *Klebsiella pneumoniae* (approximately 7%), Proteus mirabilis (5%), *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Streptococcus bovis*, and the fungus *Candida albicans* (for the remainder of the percentage) (24). UPEC populate the bladder with a range of virulence factors and hence play important roles in UTI pathogenesis. Surface structural components such as lipopolysaccharide (LPS), polysaccharide capsule, outer-membrane vesicles, outer-membrane proteins (OMPs), flagella, curli, pili, non-pilus adhesins, and secreted toxins, secretion systems, and Ton B-dependent iron-uptake receptors, including siderophore receptors, are examples of the virulence factors (32). Flagellin is a basic structural protein of bacterial flagella that is a main stimulator of the innate immune system (32). *E. coli* in human, is distinguished by several Toll-like receptor (TLRs), comprising TLR1, TLR2, TLR4, TLR5, TLR6 (7). Flagellin is a pathogenic factor that promotes both host cell adhesion and invasion. It is also a powerful immune stimulant that changes both the innate and adaptive immunity during microbial infections (14). When flagellated bacteria enter the host, flagellin is detected as a pathogen invasion signal by Toll-like receptor 5 (TLR5), triggering the innate immune response (28). Toll-like receptor 5 (TLR5) is the bacterial Flagellin receptor. According to reports, TLR5 activation aids in the fight against infections, particularly at mucosal sites, by eliciting responses from epithelial and immunological cells (26). It is important in host defense against UPEC infection because it mediates flagellin-induced inflammatory responses in the bladder that restrict bacterial proliferation in both the bladder and the kidney (21).About 23% of the general population has functional TLR gene variants that have an immunological impact (29). For example, in the TLR5 gene (1174 C>T), the rs5744168 T allele encodes a stop codon at codon 392 (TLR5r392x), leading in truncation of the TLR5 transmembrane signaling region. This nonsense polymorphism appears to be associated with the development of a several diseases, including urinary tract infections (17) ,Legionnaires disease (18), bronchopulmonary dysplasia and cancer (34), among others. Therefore, this study was aimed to investigate the role of some TLR5 polymorphism and *E.coli* flagellin gene variations with increased risk to rUTI.

MATERIALS AND METHODS

Specimens collection: One hundred and eighty blood specimens and 180 mid-stream urine specimens were collected from adult women aged 14 to 86 years during the period from the September 2020 to the Mars 2021. It was collected from Baghdad Teaching Hospital, Ghazi al-Hariri Hospital and al-Beshara laboratory. The College of Science Research Ethics Committee approved the study protocol (Reference: CSEC/1120/0011). The cases divided into two groups, the first one was patient group (120 women) with rUTI (case were diagnosis according to 3 UTI episodes within 1 year separated by at least 30 days or 2 UTIs within 6 months) and the other one was control group (60 women) who have not been infected with rUTI.

Isolation and identification of bacteria

Positive urine cultures were defined as the growth of a single colony with counts greater than 10^5 colony forming units/ml. For the initial identification of *E. coli*, all specimens were inoculated on MacConkey agar and incubated for 24 hours at 37 °C. The purple colonies were streaked on Eosin Methylene Blue (EMB) agar plates and incubated at 37 °C

for 20 hours. For the rapid isolation and probable identification of most uropathogens from culture with several micoorganisms, HiCrome UTI agar was used (10). The pure isolates were maintained in nutrient agar (HiMedia, India) for further tests.

Molecular techniques

DNA extraction: For extraction of DNA from blood cells, the gSYNC™ DNA extraction kit (Geneaid, Thailand) was used to extract DNA from a whole frozen blood sample as Manufacturer's directions (8). While for bacteria, the DNA extraction was performed by using Boiling method (1). Brefily, a 500 µl of distill water was transferred to 1.5 ml microcentrifuge tube . A loop full of bacterial culture was transferred to a distill water and well mixed using vortex, after which the specimen was centrifuged at 1,400 rpm. The supernatant was discarded and 100 µl of TE buffer was added, mixed well then incubated in water bath at 100°C for 10 min (25). After that, the sample was transferred from water bath to the freezer immediately for 20 min. The supernatant was transferred to new Eppendorf tube after centrifugation at 1200 rpm for 5 min and stored in deep freezer until use. The purity and concentration were confirmed with Nanovue plus spectrophotometer (GH Health care, USA).

Polymerase chain reaction

Amplification of *TLR5* gene:The primers used for amplification of *TLR5* gene were designed using Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgie), the forwarded primer was 5´-CTTGCCTACAACAAGATAA-3´ and the reverse primer was 5´CGTGGAAAGAGAGAAGA-3´. The entire volume of 25 µl, the PCR mixture was made up from 5µL of PCR premix (Accupower, Bionear),), 2 µl of each primer and 3 µL of template DNA, 13 µl of deionized water which was transferred to complete the rest volume. The PCR technique included an initial denaturation at 95ºC for 5 min, followed by 30 cycles of denaturation at 95ºC for 40 sec, annealing at 55.6 ºC for 35 sec, extension for 40 sec at 72ºC and final extension for 8min at 72ºC.

Amplification of *fliC*: The primers used for amplification of *fliC* in *E. coli* were 5´-AATACCAACAGCCTCTCGCT-3´ and 5´-AGAGACAGAACCTGCTGC-3´ (27). PCR mixture included 5µl of PCR premix, 1.5µl of each primer (forward and reverse), 2µl of template DNA and 14 µl of deionized water which was transferred to complete the rest volume. The PCR technique involved an initial denaturation at 95ºC for 4 min, followed by 27cycles of denaturation at 95ºC for 30 sec, annealing at 60 ºC for 40 sec , extension at 72ºC for 1 min and the final extension at 72ºC for 5 min. The amplified products for both genes were performed on a 1.5 % agarose gel labeled with Red Safe in Tris borate EDTA buffer (TBE, pH 8.4) and examined under an ultraviolet Transilluminator. The size of these products was determined using the 100 bp DNA ladder (iNtRon, Korea).

DNA sequencing

Sanger sequencing was performed to detect SNPs found in the amplified target of *TLR5* using forward primer for 99 specimens (39 from 43 patient were urine culture had *E. coli* and 60 control). Furthermore, the *fliC* PCR product of 34 isolates were sent for Sanger sequencing (forward and reverse) using prABI3730XL, automated DNA sequencer, by Macrogen Corporation-Korea. The results were received by email then analyzed using geneious software. Homology search was performed using BLASTn algorithms which are accessible at the National Center for Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov).

Ensembl genome browser was employed for search SNP related population information. Expasy translation tool was used for translation of nucleotides to amino acids residues (https://web.expasy.org/translate/) while CLUSTAL O(1.2.4) for multiple amino acids sequence alignment (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Statistical analysis

For data analysis, IBM SPSS version 26.0 was employed. The mean ± SE of mean was computed. The student T-test was also used to evaluate the probability. The probability for non-parametric data was calculated using Pearson's chi-square test. For genotyping and

allele frequencies, WinPepi version 11.65 calculated the odd ratio and Fisher's exact probability. This Hardy-Weinberg calculator is used for genotyping and estimating allele frequencies (3).

RESULTS AND DISCUSSION

From 180 specimens were collected from women of different age, Sixty of them which have been not suffering from recurrent UTI and the culture of urine was negative ,which is consider as control group. As for the rest of the urine specimens (120), they were cultured on different media and obtained 43 (35.8%) *E. coli* isolates. Four isolates have been lost while working and then only thirty nine isolates have been involved for subsequent study. Close percentage isolation findings were obtained by Al-Nassiry and Zalzala (6) with 30.4 % of isolates being *E.coli*. To confirm the identification of *E coli*, HiCrome UTI agar (Figure 1) was used which showed growth of the 39 concerned isolates in the form of pink-colored colonies and distinguish between the bacteria causing co-infection in a same rUTI patient. The chromogenic medium allows for the rapid pathogen isolation and preliminary identification of most uropathogens from culture, including diverse species (8). Chromogenic medium has demonstrated benefits such as a 20% reduction in identification time and effort, rapid detection of mixed cultures, and a decrease in the biochemical tests number performed for identification of bacteria , all of which result in a cost reduction (5). Some patients were infected by one species while the other have co-infection (infected by more than one species).Where 69.2% of cases have one infection which was the higher percent of infection, 30.8% of cases were co-infection.

Detection of *TLR5* polymorphisms

The case-control design of the current study was designed to determine that *TLR5* pathway gene variants were related to susceptibility to rUTI in adult women.. Controls and rUTI cases had a significant differences ($p \leq 0.01$) in mean age where the mean age of control were 36.13 ± 1.68, while the mean age of cases were 44.74 ± 3.05. This is due to several reasons, the important one was a common factor between case and control group which is the difficulty to find women have a UTI three times in six months so the search was focus to the elderly women, in same time this difficulty is return when collect specimens from women without UTI. The *TLR5* designed primers were successful to amplify the target gene in 99 DNA extracted blood specimens from both control and rUTI cases. The bands (924 bp) were confirmed with gel electrophoresis (Figure 2). After analysis of sequenced *TLR5* products using geneious software, four SNPs were found in the analysis segment include rs5744168 (C1174T), rs775385356 (A1669G), rs2072493 (A1775T/G) and rs5744174 (T1846C).

Figure 1. *E. coli* isolated from rUTI on HiCrome UTI agar

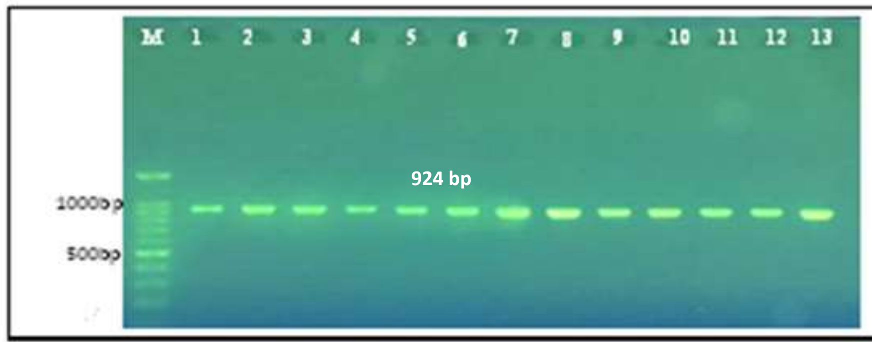

Figure 1. Amplification of *TLR5* (924bp) in patient and control group, PCR products in gel electrophoresis. Agarose 1.5%, 75 V/cm for 45 min, dyed with a non-hazardous red stain and examined with an ultraviolet transilluminator. Lane 1-7: Amplicons of *TLR5* in control group. Lane 8-13: Amplicons of *TLR5* in patient group M: 100 bp DNA ladder

The results of *TLR5* genotyping SNPs were compared to the list of global genetic mutations in *TLR5* from the 1000 Genome Project (http://www.ensembl.org) as shown in figure 3 . The frequencies of population noticed in this study for rs5744174, rs2072493 and rs5744168 were in nearby to frequencies reported from American, European and South Asia populations respectively as well as the distributions of genotype frequencies was reduced when comparing with the population in Africa. While rs775385356 showed a notably higher frequency in this study population compared with frequency less than 0.001% (very rare) and no population genetics registered in Ensembl genome browser. The most frequent allele in the Iraqi population of this study was the G (C in reverse strand) of rs5744174 SNP with 0.34 frequency. Whereas the T allele (A in reverse strand) of rs2072493 SNP and G allele (C) of rs5744168 SNP, occurred at a frequency of 0.16 and 0.09 respectively.

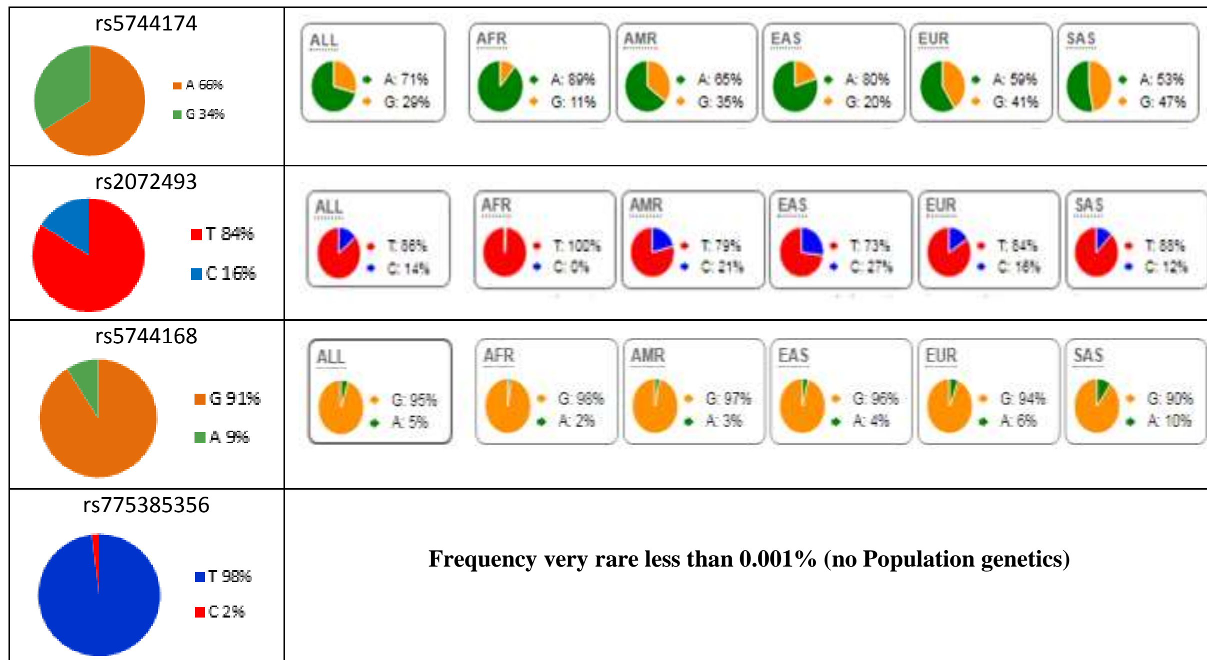

Figure 2. *TLR5* SNPs in this study were compared to the list of global genetic mutations in *TLR5* from the 1000 Genome Project (http://www.ensembl.org)

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notably higher frequency in this study population compared with frequency less than 0.001% (very rare) and no population genetics registered in Ensembl genome browser. The most frequent allele in the Iraqi population of this study was the G (C in negative strand) of rs5744174 SNP with 0.34 frequency. Whereas the T allele (A in negative strand) of rs2072493 SNP and G allele (C in negative strand) of rs5744168 SNP, occurred at a frequency of 0.16 and 0.09 respectively. Statistical analyses of data examined whether studied SNPs of *TLR5* were associated with susceptibility to rUTI. Genotype and allele frequencies of the four investigated SNPs (rs5744174, rs2072493, rs5744168 and rs775385356) observed no significant variation (p> 0.05) from Hardy-Weinberg equilibrium (HWE) achieving the equilibrium between rUTI and control cases (Table 1). *TLR5*-C1174T is a polymorphism that codes for a stop codon variant that cancels flagellin signaling. It found previously that T allele of rs5744168 works in a predominant model regarding to allele C when investigated this SNP with susceptibility to legionnaires' disease (18). Our findings on *TLR5*-C1174T differed with those of Hawn *et al*. (19), who found that the *TLR5*-C1174T SNP was related to increased susceptibility to recurrent UTI in adult women. Although the frequencies of CT and TT gentoypes in Hawn study were non-significant to control, it appeared significant when compared genotype frequencies with a predominant model. Further, *TLR5*-A1775T/G *TLR5*-T1846C polymorphisms were non-significant.

Table 1. Genotypes and alleles frequency of *TLR5* SNPs in women with rUTI versus controls

TLR5 SNPs	Allele/ Genotype	rUTI cases N=39		Control cases N= 60		OR	CI(95%)	*p*-value
		N	%	N	%			
rs5744168	*C*	113	94	73	96	0.66	0.17-2.63	0.743
	T	7	6	3	4	1.51	0.38-5.97	
	CC	35	91.11	53	88.33	0.65	0.16-2.64	0.736
	CT	3	7.89	7	11.67	1.54	0.38-6.26	0.736
rs775385356	*A*	70	0.59	50	0.66	0.76	0.42-1.38	0.449
	G	48	0.41	26	0.34	1.32	0.73-2.39	
	AA	25	65.79	34	57.63	0.71	0.31-1.63	0.524
	AG	0	0.0	2	3.39	3.35	0.16-69.0	0.518
	GG	13	34.21	23	38.98	1.23	0.53-2.85	0.672
rs12072493	*A*	105	0.89	69	0.91	0.82	0.31-2.14	0.811
	G	13	0.11	7	0.09	1.22	0.47-3.19	
	AA	32	84.2	47	79.7	0.73	0.30-2.13	0.790
	AG	5	13.2	11	18.6	1.51	0.49-4.70	0.581
	GG	1	2.6	1	1.7	0.64	0.04-10.2	1.0
rs5744174	*T*	60	0.51	44	0.58	0.75	0.42-1.34	0.378
	C	58	0.49	32	0.42	1.33	0.75-2.37	
	TT	16	42.1	19	32.2	0.65	0.28-1.51	0.388
	TC	12	31.6	22	37.3	1.29	0.55-3.03	0.665
	CC	10	26.3	18	30.5	1.23	0.50-3.02	0.819

rUTI= recurrent urinary tract infections, OR= odds ratio, CI= Confidence interval

In pediatric study on renal parenchymal infections, found no statistical correlation between examined *TLR5* SNPs (rs5744174, rs2072493 and rs5744168) allele frequencies with the acute pyelonephritis and acute lobar nephronia versus control groups (7). In other study on association of *TLR5* rs5744168 with febrile UTI, found neither of the alleles or genotypes was related with febrile UTI. The data were re-analysed after removal of the individuals with recognized urinary tract disorders or chronic renal insufficiency which might mask genetic effects resulting in show association between rs5744168 and protection from UTI (p = 0.011) (34). TLRs are essential for microbial recognition and act as sensitive sensors for microbial attack, also work as effectors for innate immunity dependent on TLR. This allows to eliminate pathogens in the host that would else cause disease or death. Many researches have mentioned that *TLR* SNPs can influence the ability of individual to react to TLR ligands, resulting in susceptibility changing to infections or inflammation. The

changing susceptibility can be decreased the inflammatory response, such as take places in asymptomatic bacteriuria, rUTIs and protection with pyelonephritis. Otherwise, immune response can be an exaggerated leading to severe infection, as happens in severe atopic dermatitis and tuberculosis (7). There are many potential mechanisms for the role of TLRs in the pathogenesis of lower and upper urinary tract infection. If the bladder and kidney epithelial cells have a TLR-signaling defect, early identification of *E. coli* bacteria would be eliminated, and signaling pathways may be delayed. Furthermore, recruited neutrophils or monocytes to the bladder may have altered responses, increasing susceptibility to UTIs. Potential explanations for contrast in findings might be return to the sample size that has statistical force to detect considerable associations with genetic polymorphisms, ethicinity and polymorphism frequency as found that CT and TT genotypes frequencies for rs5744168 was 11.7% in our population control vs. 7.4% in Caucasian American women in Hawn *et al*. (19) study and 15.8% in Netherlands study done by van der Starre *et al*. (34). The same SNPs (rs2072493, rs5744174 and rs5744168) were investigated in 77 patients infected with *H. pylori* and 230 healthy persons. The results revealed that these variations are lower frequent in Tamils (south Indian) and failed to present a significant risk for developing chronic *H. pylori* infections (15). Furthermore, several investigation were done to determine whether *TLR5* rs5744168 with susceptibility to or protection from systemic lupus erythematosus (SLE) which indicated no significant relation of rs5744168 with Caucasian American (11) and in Guangxi Zhuang and Han, China SLE subjects (37). Further, the same SNP showed neither allelic or genotype association found in rs5744168 with SLE. Although, the frequency of T allele and TT genotype were increased significantly in the lupus nephritis group (12).

Amplification and sequence of *E. coli fliC*

Flagellin (encoded by *fliC*) in motile bacteria is an important and universal subunit of flagella. Almost all 53 H-serotypes are located in *fliC* locus. The *fliC* of 34 (87.2%) *E. coli* isolated from rUTI were amplified using specific primers. Nearly all *E. coli* isolates made a single band varying in size (between 1000bp and 2000 bp) (figure 3). Some isolated stay amplified a non specific bands although the reaction conditions were optimized several times. In addition, five (12.8%) isolates were failed to interact with the *fliC* primer. Previously, several studies noticed that amplification of *fliC* resulting different amplicon size reflect the H- type of flagella belong it (21, 23, 30). The reasons why not *fliC* were amplified in all isolates might be due to some of H-antigen genes are at loci other than *fliC* such as *flnaA*, *fllA*, *fmlA* or *flkA* discovered in *Salmonella* species which act as alternative flagellar phase (22). It was found that 34 out of 53 H types (13, 20, and 50 not in use) in *E. coli* are expressed by genes at the fliC locus, with the remaining ten encoded by other loci or due to the fliC gene being at least partly deleted (33).

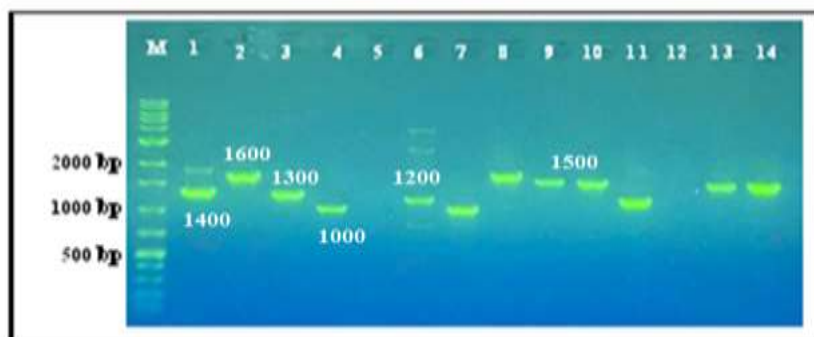

Figure 3. Amplification of *fliC* (1000-2000 bp), PCR products in gel electrophoresis from *E. coli* isolates from rUTI patients. Agarose 1.5%, 75 V/cm for 45 min, dyed with a non-hazardous red stain and examined with an ultraviolet transilluminator. Lane 1-4, 6-11, 13-14: Amplicons of *fliC*. Lane 4,7 1000 bp, lane 6,11 1200 bp, lane 3,8 1300 bp, lane 1,13,14 1400 bp, lane 9,10 1500 bp, lane 2 1600 bp, lane 5 and 12 represent failed the amplification of *fliC* in *E. coli* isolates. M: 100 bp DNA ladder

The *fliC* sequence was obtained for 28 isolates whilst the rest (6 isolates) were failed to be sequenced. Using the NCBI's Basic Local Alignment Search Tool (BLAST), found the isolates were belonged to 12 flagellar antigen serotype when alignment their *fliC* sequences with GenBank strains (table 2), the sequencing findings revealed 95-100 percent compatibility with reference strains. Comparison of nucleotide sequence for studied *fliC* demonstrates height similarity in both 5' and 3' regions which indicates the *fliC* termini from different flagellin serotypes of *E. coli* are conserved. On the other hand, the middle region was variable had highly various polymorphisms (418 polymorphism, 147 of them were missense and the rest were silent). By using Expasy translate tool and Clustal Omega, the analysis of *fliC* amino acid sequences of 11 H-serotypes (the rest serotype H34 did not contain the entire sequence for the translating it into a protein) resulted in proteins of different lengths ranging between 324-634 residues (figure 3). The conserved region in N-terminal expands the first 170 residues while the C-terminal domain extends approximately 75 residues. In contrast, the central region was variable poorly preserved have multiple alignment gaps. The results showed list of conserved amino acids in both FliC termini previously mentioned in many investigations were essential in TLR5 recognition. L89, Q90,R91, L95, Q98, N101 and E115 in N-terminus instead of L88, Q89,R90, L94, Q97, N100 and E114 reported in other studied which already exists without substitutions in all studied serotypes. On the other hand, the conserved amino acids in C-terminus (I411, L415, T420, L425 D412, R431 and N440) were observed in some serotypes. Further, the N277 residue that reported its central role for TLR5 interaction (10) were found in H9(N277), H6, H10, H12(N276), H52(N274), H9(N277), H5, H25 (N273) and the rest serotype had substitutions in this position.

Table 2. *E. coli* rUTI isolates, *fliC* PCR amplicon sizes and ID of sequencing in GenBank

Local Isolates	Approximate Amplicon size(bp) in agarose gel	Serotype	Sequence ID in GenBank
EcoF 3, 11, 29, 33	1000	UPEC	CP054232.1
EcoF 10,19,28	1300	H5	AY249990.1
EcoF 1,6,7,16,34,35,36	1600	H6	AY249991.1
EcoF 2	2000	H9	AY249994.1
EcoF 13,20,21,22,27,37	1200	H10	AY249995.1
EcoF 31	1700	H12	AY337471.1
EcoF 5	1200	H25	LR134226.1
EcoF 26	1700	H30	AY250011.1
EcoF 24	1600	H31	AY250013.1
EcoF 39	1600	H34	AY250016.1
EcoF 25	1300	H38	AY250018.1
EcoF 14	1300	H52	AY250028.1
EcoF= *E.coli fliC*			

The similar results were mentioned in various studies, they reported that the termini (N and C) of flagellin in *E. coli* are extremely conserved and vital for the flagella structure whilst the middle region can be quite variable and encodes for parts of the protein that are surface-exposed and H-type specific epitopes (20, 33). The variation in length of amino acids was observed not effect on flagellin function as in H7 serotype flagellin had shorter amino acids sequence in 11 residues than H12 but didn't effect diameter of filament or its architecture. However, the deletion it might influence morphology of filament and H antigenicity. Further, mutations in terminal-region have lead to straight, nonfunctional filaments (35). The flagellin is recognized specifically by TLR5 (38). D1 domain are very essential for bacterial motility, therefore removing of the 99 residues from N-terminus 99 and 416–444 residues in C terminus of FliC of the *S. typhimurium* resulting in inhibited of TLR5 recognition, whilst the D3 domain is base for evade antibody responses through structural heterogeneity. Studying deeper on 22 mutants in *S. typhimurium* flagellin found ten of them (L88, Q89,R90, L94, Q97, E114, I411, L415, T420 and L425) and three (N100, D412 and R431) were significantly reduced76-97% and 50–75% recognition of TLR5

respectively (36). The R90 and Q97 were also with N440 mentioned as key residues in in TLR5 recognition after studying *S. enterica*. The R90 has an substitution (R90S) while Q97 and N440 are conserved. Further, molecular docking to FliC react with TRL5 appeared that *S. enterica* had the strongest whilst *E. coli* had the weakest interact with TLR5 (13). In conclusion, it was no relation between genotypes or allele frequencies with *fliC* polymorphisms found in *E.coli* isolated from rUTI.

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