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Expression of *flu* gene in Ciprofloxacin resistant uropathogenic *Escherichia coli*

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Abstract

Uropathogenic E. coli (UPEC) exhibits various virulence factors that enable it to both establish itself in the urinary tract and trigger disease symptoms. Ag43, which is encoded by the *flu* gene, promotes bacterial adherence and the production of biofilms on human urothelial cells. Using the Kirby-Bauer method, the ciprofloxacin sensitivity of forty UPEC isolates was assessed. Twenty-two UPEC isolates (21 of which were resistant to ciprofloxacin and one of which was sensitive) were used in order to calculate the MIC of ciprofloxacin using the macro tube assay. Using the (ABIO pure extraction) procedure, genomic DNA was extracted from bacterial growth. Real time PCR (qRT-PCR) proved successful in detecting the expression of the *flu* gene. Antimicrobial susceptibility testing of UPEC isolates showed that 21 out of 40 isolates (52.5%) were resistant to ciprofloxacin. MIC for isolates resistant to ciprofloxacin was ≥ 64 (µg/ml). The identification of the *flu* gene by the analysis of PCR showed that (21/22; 95.45%) carried the *flu* gene. The findings of qRT-PCR revealed that (4/11; 36.3%) of the UPEC isolates had downregulation of the *flu* gene, whereas (7/11; 63.6%) showed overexpression. The *flu* gene (folding=5.59) had the highest expression in UPEC isolate number 3. A significant correlation was observed, with most UPEC isolates showing overexpression of the flu gene. Thus, the *flu* gene plays a crucial part in the pathogenicity of isolates from UPEC.

Keywords: UPEC, *flu* gene, Real Time PCR, Ciprofloxacin

التعبير عن جين *flu* في الممرضة البولية *E.coli* المقاومة للسبروفلوكساسين

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الخلاصة

تُظهِر الإشريكية القولونية المسببة للأمراض البولية (UPEC) عوامل فوعة مختلفة تمكنها من تثبيت نفسها في المسالك البولية وتسبب أعراض المرض. يعزز Ag43، الذي يتم ترميزه بواسطة جين flu، الالتزام البكتيري وإنتاج الأغشية الحيوية على الخلايا البولية البشرية. باستخدام طريقة الاقراص، تم تقييم حساسية السيبروفلوكساسين لأربعين عزلة من UPEC. تم استخدام 22 عزلة (21 UPEC منها كانت مقاومة للسيبروفلوكساسين وواحدة حساسة) من أجل حساب MIC للسيبروفلوكساسين باستخدام اختبار الأنابيب. باستخدام طريقة (ABIO)، تم استخراج الحمض النووي الجينومي من نمو البكتيريا. أثبت تفاعل البوليميراز المتسلسل (PCR) في الوقت الحقيقي (qRT–PCR) نجاحه في اكتشاف التعبير عن جين flu. أظهر

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اختبار الحساسية المضادة للميكروبات لعزلات UPEC أن 21 من أصل 40 عزلة (52.5٪) كانت مقاومة للسيبروفلوكساسين. كان الحد المثبط الأدنى للعزلات المقاومة للسيبروفلوكساسين ≤ 64 (ميكروجرام/مل). وأظهر التعرف على جين flu عن طريق تحليل PCR أن (22/21؛ 95.45%) تحمل هذا الجين. كشفت نتائج qRT–PCR أن (11/4؛ 36.3٪) من عزلات UPEC كان لديها تناقص في تنظيم جين flu في حين أن (11/7؛ 63.6٪) أظهرت فرط التعبير. كان لجين flu (المطوي = 5.59) أعلى تعبير في عزلة UPEC رقم 3. ولوحظ وجود ارتباط كبير، حيث أظهرت معظم عزلات UPEC زيادة في التعبير عن جين flu. وهكذا، يلعب هذا الجين دورًا حاسمًا في التسبب في إمراضية المعزولات من UPEC.

1.Introduction

Urinary tract infections, which rank among the most common illnesses in numerous countries, are predominantly caused by the Gram-negative bacterium Escherichia coli [1, 2]. Numerous fluoroquinolones, such as ciprofloxacin and norfloxacin, ofloxacin, levofloxacin, gatifloxacin, and lomefloxacin, are currently licensed for the treatment of UTIs [3, 4]. Within this pathotype, there is a significant increase in antibiotic resistance [5]. *E. coli* has become more resistant to fluoroquinolone antibiotics like ciprofloxacin during the past ten years. In Switzerland, a 10-year testing of urine *E. coli* samples exhibited an expand in ciprofloxacin resistance from 1 to 16%, while in Bangladesh (240/287; 95%) of *E. coli* isolates were resistant to Ciprofloxacin [6]. Uropathogenic E. coli (UPEC) harbors several virulence factors that contribute to its ability to colonize the urinary tract and cause infection. These virulence factors are related to UPEC's pathogenic potential [7, 8]. Genes encoding adhesions, pili, afimbrial adhesions and curli fimbriae, which disport a part in the colonization of bacteria in the urinary tract and installation of UTIs, besides genes encoding toxins that have role in the pathogenicity of these strains, are some [9-11]. Adhesin Ag43, which is expressed on the outer membrane of *E. coli* and is encoded by the *flu* gene, facilitates bacterial adhesion and the formation of biofilms on host urothelial cells, leading to inadequate pathogen clearance [12,13]. Two chemically and immunologically distinct polypeptides a and b are present in the antigen 43 complex, apparent molecular weights of 53 kDa for b and 50 to 60 kDa for a, respectively. They happen with the same stoichiometry [14]. Bacterial cells in biofilms are enclosed in a sophisticated extracellular matrix that may contain proteins, nucleic acids, and polysaccharides. (Type 1, F1C, S) pili, and F9 fimbriae, besides the adhesive autotransporter proteins are UPEC-closely related adhesins that have been linked to the development of biofilms [15-17]. UPEC can further stimulate biofilm development by utilizing flagella and thin proteinaceous structures called curli. UPEC may become less responsive to host defenses like neutrophils and antibiotics as a result of the elaboration of biofilms within urinary catheters and in connection with host tissues [18, 19]. The aim of the present study was to find the *flu* gene expression in UPEC isolated from different UTI patients.

2.Materials and Methods

Bacterial isolate

A study collected 200 urine samples from patients with suspected urinary tract infections (UTIs). These samples were used to isolate E. coli through biochemical identification and by using specialized agar media - Hichrome E. coli and Hichrome UTI agar from Himedia, India [20]. The compact VITEK 2 system was used to ensure the results of bacterial identification. This research project was approved by the Biology Department Ethics Committee at the College of Science, University of Baghdad on September 21, 2022 (Ref.: CSEC/0922/00772023) in accordance with Iraqi laws and regulations.

Susceptibility of UPEC

The susceptibility of 40 UPEC isolates to ciprofloxacin and 15 other antibiotics was assessed using the Kirby-Bauer disk diffusion method and the VITEK 2 Compact automated system [21-22]. Macro tube assay was employed to examine the sensitivity of the UPEC isolates and estimate the MIC of Ciprofloxacin. Bacterial cells were diluted to 1.5×10^8 (cell/ml) in comparison to McFarland tube, then (10µl) inoculated on Mueller Hinton broth containing Ciprofloxacin with double serial concentrations (1ml). The MIC value is represented by the first clear test tube following turbid tubes after the tubes are incubated for 24 hours [23-24].

Detection of *flu* gene by PCR

Genomic DNA was extracted from bacterial growth according to the protocol of (ABIO pure Extraction) [25]. The amount of DNA extracted was measured using a Quantus Fluorometer. Diluted Quantifluor Dye was combined with DNA. The *flu* gene was detected in 22 *E.coli* isolates amplified by PCR using certain primer (Macrogen, Korea) as shown in **Table 1.** After amplification, PCR yields were determined.

Expression of *flu* gene

RNA Purification

RNA extraction from the sample was performed using the TRIzol™ Reagent method. The process began with cell lysis and centrifugation to obtain a cell pellet. Then, 0.75 mL of TRIzol™ Reagent was added to this pellet. The lysate was mixed with chloroform, then centrifuged for ten minutes at 12,000 rpm. The mixture separated into three distinct phases: an upper aqueous layer, a lower organic layer, and an interphase layer. A fresh tube was filled with the RNA-containing aqueous phase. Isopropanol was added, incubated for 10 minutes, then centrifuged to precipitate RNA. A white, gel-like pellet of precipitated total RNA formed at the base of the tube. Following precipitation, the RNA was washed and then treated with 70% ethanol. The pellet was then air-dried. For RNA solubility, the pellet was rehydrated in Nuclease-Free Water and incubated for 10 to 15 minutes at 55 to 60°C in a water bath [26]. A Quantus Fluorometer was employed to determine the concentration of extracted RNA in order to evaluate the quality of samples. After mixing RNA with QuantiFlour Dye and letting it sit at 25 ºC for five minutes, the concentration of the RNA was measured. Real-time PCR (qRT-PCR) and conventional PCR were conducted using specific primers from Macrogen, Korea, as shown in **Table 1.**

Table 1: PCR Primer Selection: Conventional PCR and Real-Time qRT-PCR Options

Gene	{5' → 3'}	Annealing Tm	Product size bp	Reference
flu	F: CGGCGGGCAATGGGTACA R: CAGCTCTCACAATCTGGCGAC	55	383	13
E.co_16s	F:GTTAATACCTTTGCTCATTGA R:ACCAGGGTATCTAATCCTGTT	60	340	27

Real time PCR (qRT-PCR) program

Table 2 shows the qRT-PCR procedures, temperature, duration, and number of cycles utilized to determine the expression of the *flu* gene.

Table 2: (qRT-PCR) program

Steps PCR	**Temp** ºC	**Time**	**Cycle**
RT Enzyme Activation	37	15 m	1
Initial Denaturation	95	5 m	
Denaturation	95	20 s	40
Annealing	55 or 60	20 s	
Extension	72	20 s	

Analysis the expression of *flu* gene

Determine the relative quantity of the *flu* gene using the Livak Method [28]

Folding $=2^{-\Delta\Delta CT}$

$\Delta CT = CT_{\text{gene}} - CT_{\text{House Keeping gene}}$

$\Delta\Delta CT = \Delta CT_{\text{Treated or Control}} - \Delta CT_{\text{Control}}$

3.Results and Discussion

Susceptibility of UPEC isolates

The results obtained by using the Kirby-Bauer method and VITEK 2 Compact system for the susceptibility of UPEC isolates were obtained and susceptibility the results of isolates testing showed that 4 out of 40 (10%) were sensitive to all antibiotics tested. 21 isolates (52.5%) were resistant to ciprofloxacin. The highest resistance among UPEC isolates was observed for ampicillin (34/40, 85%) and cefazolin (33/40, 82.5%). Meanwhile, resistance was lowest or nil for amikacin and tigecycline (0/40, 0%). Mahdi et.al.(2023) reported that highest resistance in *E.coli* isolated from Tigris River was to ampicillin (55%), This result corroborated our findings and those of study [29], which mentioned penicillins as having a considerable resistance ratio among antibiotics among isolates responsible for recurrent urinary tract infections [30]. Results from Sabri and Kareem (2020) that matched ours appeared that 66% of the *E. coli* isolates from UTIs were resistant to ciproflaxcine [31]. Another study showed that the rates of resistance among *E. coli* isolates engaged in the treatment with azithromycin. Resistance rates to ciprofloxacin and ofloxacin were reported at 28.5% and 60.7%, respectively, while ciprofloxacin resistance was found in 42.8% of cases [32]. Moreover, 76% of the 50 UPEC isolates tested exhibited resistance to ciprofloxacin [33]. According to these results, ciprofloxacin resistance spread by UPEC and associated with communicable UTIs was connected to multidrug resistance [34].

Determining of Minimum Inhibitory Concentration (MIC)

The macro test tube was employed to determine the MIC for Ciprofloxacin and the sensitivity of UPEC isolates. The findings showed that the MIC for isolates resistant to ciprofloxacin was ≥ 64 (µg/ml), whereas the MIC for ciprofloxacin was 0.031 (µg/ml) in the one selected isolate that was susceptible to all antibiotics as shown in **Table 3**.

Table3: MIC Distributions of Ciprofloxacin against Uropathogenic *E. coli* Isolates

UPEC isolates	**Susceptibility to Ciprofloxacin**	**MIC value(µg/ml)**
1	sensitive	0.031
2	Resistant	32
3	Resistant	128
4	Resistant	1024
5	Resistant	256
6	Resistant	64
7	Resistant	256

8	Resistant	32
9	Resistant	64
10	Resistant	32
11	Resistant	256
12	Resistant	64
13	Resistant	128
14	Resistant	64
15	Resistant	256
16	Resistant	1024
17	Resistant	64
18	Resistant	64
19	Resistant	64
20	Resistant	64
21	Resistant	256
22	Resistant	128

Due to its easy absorption from the gastrointestinal system following oral administration, ciprofloxacin is the most often used medicine for treating urinary tract infections. It is a first-line treatment for a variety of illnesses. Broad-spectrum coverage and high urine excretion rates are two further benefits of ciprofloxacin. Once administered, supra-inhibitory concentrations of antibiotics in the body gradually decrease to sub-minimum inhibitory concentrations (MICs) over time [35]. Mohsin (2022) Reported that the MIC was (0.250 or 1 µg/ ml) of Ciprofloxacin in sensitive *E.coli* isolates [24]. In another study the isolates of *E. coli* were subjected to doubling dilution tests with all antimicrobials have MIC in a range of 0.0625 to 32 or 64 µg/mL [36]. MIC value for ciprofloxacin. 62% of 70 *E. coli* isolates were found to have MIC values more than 4 mg/l, meaning they fell into the resistant category [37]. Complex and diverse mutations are linked to both low- and high-level ciprofloxacin resistance. Ciprofloxacin minimum inhibitory concentration (MIC) seemed to be correlated with the kind and quantity of mutations found in 15 clinical *E. coli* isolates. [38].

Detection of *flu* gene by PCR

PCR analysis visualized on agarose gel enabled the detection of the *flu* gene in 22 UPEC isolates. Of these, 21 were ciprofloxacin-resistant, while one was sensitive to all antibiotics tested. Results showed that 21 (95.45%) of the 22 UPEC isolates carried the *flu* gene Figure 1.

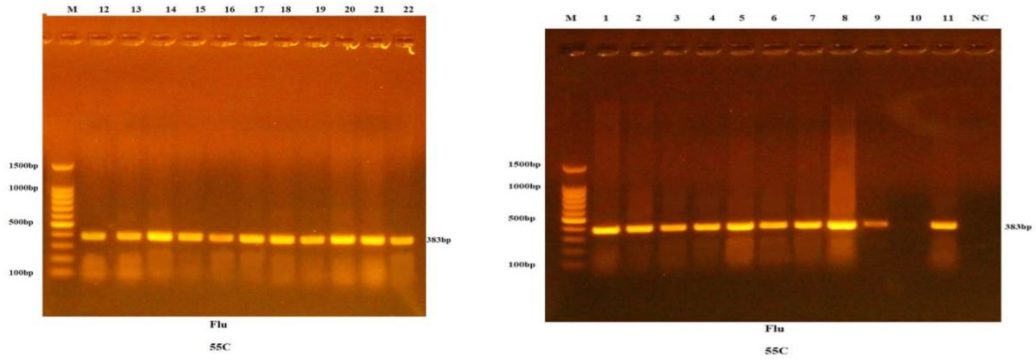

Figure 1: Results of the amplification of flu gene of E. coli samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes (1-22) resemble 383bp PCR products.

Machuca *et. al,* discovered that the *flu* locus produced a product that was identical to antigen 43 (Ag43), a protein located on the outer membrane [39]. Many different strains of *E. coli* express Ag43. It's interesting to note that numerous pathogenic strains display it. Ag43-Ag43 intercellular contact is demonstrated to be necessary for auto aggregation. Accordingly, an assess of *E. coli* strains associated with urinary tract infections and enter pathogenicity exhibited that 77% and 60%, respectively of these organisms were able to express Ag43 [40]. AL- Abady Y. and AL-Hashimy A. (2021) revealed that the *flu* gene was present in 75% of the 20 UPEC isolates in total [13]. Additional research showed that 83% (30/36) of UPEC strains had the *flu* gene, whereas 56% (35/62) of nonpathogenic *E. coli* strains did [41]. These outcomes supported our hypothesis that a large percentage of the *flu* gene is present in UPEC isolates.

Expression of *flu* gene by Real time PCR

A novel molecular approach called Real time PCR (qRT-PCR) [42,43] employing a specific primer (the housekeeping gene of (16srRNA) of *E.coli* proved successful in detecting the expression of the *flu* gene after treating (11) selected UPEC isolates with sub-MIC value of Ciprofloxacin for each isolate. The accuracy of gene product amplification was assessed using cycle threshold (Ct) values from reactions of both the housekeeping gene and flu gene. The values for dCt, ddCt, and fold change were calculated following the method described by Livak and Schmittgen [28], as presented in **Table 4**. The findings revealed that (4/11) (36.3%) of the UPEC isolates had downregulation of the *flu* gene, whereas (7–11) (63.6%) showed overexpression. The *flu* gene (folding=5.59) had the highest expression in UPEC isolate number 3.

Table 4: The Results of Quantitative Real-Time PCR Analysis of Housekeeping Genes and *flu* Gene

	Sample	16s*E.coli*	*Flu*	dct	ddct	Folding
1	1	15.55	22.64	7.09	0.47	0.72
	C1	19.55	26.17	6.61	0.00	1.00
2	2	12.52	19.92	7.40	-0.08	1.06
	C2	11.48	18.96	7.48	0.00	1.00
3	3	11.87	17.09	5.23	-2.48	5.59
	C3	12.56	20.27	7.71	0.00	1.00
4	4	8.92	14.10	5.18	-0.28	1.22
	C4	10.60	16.07	5.46	0.00	1.00
5	5	13.27	19.48	6.21	0.91	0.53
	C5	8.00	13.30	5.30	0.00	1.00
6	6	6.48	13.33	6.85	-0.45	1.36
	C6	8.38	15.69	7.30	0.00	1.00
7	7	11.43	16.91	5.48	-0.43	1.35
	C7	8.83	14.74	5.91	0.00	1.00
8	8	9.20	14.07	4.87	-0.20	1.15
	C8	11.00	16.07	5.07	0.00	1.00
9	9	9.58	15.32	5.74	1.22	0.43
	C9	10.27	14.79	4.52	0.00	1.00
10	10	11.60	16.67	5.07	-0.75	1.68
	C10	9.98	15.80	5.82	0.00	1.00
11	11	8.95	15.13	6.18	0.26	0.84
	C11	11.79	17.71	5.93	0.00	1.00
	C=control					

When bacteria adhere to a surface, they frequently multiply to form increasingly intricate microcolony structures. This technique is frequently made easier by auto-aggregation factors. For example, autoaggregation and the production of microcolonies in *Escherichia coli* have been linked to several variables, such as curli, fimbriae, and Ag43 [44, 45].

The impact of ciprofloxacin at sub-MIC on the expression of genes linked to biofilms, such as (*icaA, altE,* and *sigB*), as well as the biofilm composition of five pathogenic isolates of *S. epidermidis*, were investigated. Three of the five examined isolates showed an enlargement in (*sigB* and *icaAmRNA*) levels in response to 0.5 mg/l MIC ciprofloxacin. All isolates showed an increase in *altE* gene expression at the same time [29,46]. Boer et.al. (2015) discovered a correlation between the ciprofloxacin's therapeutic efficacy and its MIC in UPEC isolates. For isolates with a ciprofloxacin minimum inhibitory concentration (MIC) of less than 1 mg/L, over 94% of the infections responded successfully to ciprofloxacin therapy. The response was 32% for these isolates with a ciprofloxacin MIC ≥ 4 mg/l. Patients with MICs in the intermediate and resistant range were those who did not reply to the Ciprofloxacin treatment [36]. These results corroborated our findings; the MIC of the Ciprofloxacin-resistant UPEC isolates was ≥ 64 mg/l, which consequently elevated the expression of a gene linked to biofilm.

Conclusion

The auto-aggregation observed in our results can be attributed to Ag43, a byproduct of the flu gene. Notably, we found a high degree of correlation (95.45%) between the flu gene and auto-aggregation, with the majority of UPEC isolates exhibiting overexpression of the flu gene. Consequently, the flu gene plays a critical role in the pathogenicity of isolates from UPEC by increasing the ability of bacteria to form biofilm, which increases the resistance of bacteria to Ciprofloxacin treating.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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