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Detection of Efflux Pumps Gene and Relation with Antibiotics Resistance in Uropathogenic *Escherichia Coli* (UPEC) Isolated from Patients with Cystitis

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Abstract

In recent years, the multidrug-resistant (MDR) *Escherichia coli* has increased in urinary tract infections (UTIs). One of the highly distributed chromosomally encoded traits of resistance is efflux pump. The study intended to investigate the most common members of 5 classes of efflux pumps among uropathogenic *E. coil* isolates. *E. coli* is confirmed by green metallic sheen on eosin methylene blue and by PCR using primer for *uidA* gene. Using disc diffusion method, an antibiotic susceptibility test was peformed against 25 antibiotics. Efflux pump genes were examined via polymerase chain reaction. Biofilm formation was investigated by a 96 well polystyrene microtiter plate. All 50 isolates were resistant to amoxicillin, ceftazidime and cefotaxime, while all isolates were sensitive to imipenem. 19 (38%) *E. coli* isolates were non-MDR while 31 (62%) were MDR. The frequency rates of different efflux pump genes ranged from 66% to 100%. The biofilm formation results indicated that 98% of isolates were biofilm former while 2% were non-biofilm. The current study concludes that all efflux pumps may be highly associated with resistance to amoxicillin, cefotaxime and ceftazidime. Additionally, biofilm formation was found to be highly related to the presence of efflux pumps genes.

Keywords: Efflux pump, *Escherichia coli*, Antibiotic resistance, UPEC, UTI

التحري عن جينات مضخات الدفق وعلاقتها مع المقاومة للمضادات الحياتية في الاشريشيا القولونية الممرضة للمسالك البولية المعزولة من مرضى التهاب المثانة

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الخلاصة:

في السنوات الاخيرة لوحظ زيادة في انتشار بكتريا الاشريشيا القولونية متعددة المقاومة للمضادات الحياتية في التهابات المسالك البولية. وتعد المقاومة المتواسطة بمضخات التدفق المحموله كروموسوميا واحدة من اهم اليات المقاومة للمضادات الحياتية. تهدف الدراسة الحالية الى التحري عن خمسة مجاميع من مضخات التدفق في بكتريا في الاشريشيا القولونية الممرضة للمسالك البولية. تم تشخيص البكتريا من خلال ظهور البريق الاخضر الفلزي نتيجة لوجود صبغة يوزين ميثيلين الأزرق في الوسط، وكذلك بتقنية تفاعل البلمرة المتسلسل باستخدام بادئات موروثة ال *uidA* كما واجري فحص المقاومة للمضادات الحياتية ل 25

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مضاد باستخدام طريقة الانتشار من الاقراص. تم التحري عن مورثات مضخات التدفق بواسطة تقنية انزيم البلمره المتسلسل بالاضافة الى اجراء فحص التحري عن تكوين الاغشية الحيوية باستخدام طريقة صفيحة ال 96 حفرة. اظهرت النتائج مقاومة كل ال 50 عزلة لمضادات ال amoxicillin وceftazidime و cefotaxime في حين انها اظهرت حساسية لمضاد ال imipenem. كانت 19 عزلة غير متعددة المقاومة للمضادات بينما ال 31 عزلة المتبقية كانت متعددة المقاومة. تراوحت نسبة وجود موروثات مضخات التدفق من 66% الى 100% من العزلات وكانت نسبة العزلات المكونة للاغشية الحيوية 98% والغير مكونة 2%. نستنتج من الدراسة الحالية الارتباط الكبير بين وجود موروثات مضخات التدفق مع المقاومة لمضادات ال amoxicillin وceftazidime وcefotaxime وكذلك ارتباط مضخات التدفق بتكوين الاغشية الحيوية.

Introduction

Cystitis can be defined as one of the different types of urinary tract infections (UTIs). It is an infection that is related to lower urinary tract, in particularthe bladder [1]. By the age of 24 years, one third of females have a UTI, and one-half by the age of 32 years. Spermicide use, sexual intercourse, a previous UTI, a sex mate during the last year and a family history of UTIs are all factors that are increasing the risks of uncomplicated cystitis. Acute cystitis is far more prevalent as compared to pyelonephritis, with 18-28 episodes of cystitis regarding each pyelonephritis episode [2]. The major etiologic agent in the uncomplicated UTIs in females is uropathogenic *E. coli* (UPEC), which accounts for 75-95% of cases [3]. Antimicrobial resistance is also considerably more common in cystitis. Extended-spectrum beta-lactamase (ESBL) harbouring, fluoroquinolone-resistant and carbapenem-resistant bacteria are all examples of significant resistant organisms [4]. Multidrug-resistant (MDR) bacteria have emerged as a main public health concern around the world [5]. Efflux pumps are regarded as one of the main antibiotic resistance mechanisms, particularly in MDR-UPEC. Through extruding drugs out of the cytoplasm, efflux pumps in Gram-negative bacteria compromise the drug antibacterial efficacy. Drug efflux lowers their intracellular concentration, rendering the pathogens to become more resistant to the drug [6]. The major facilitator superfamily (MFS), small multidrug resistance (SMR) family, ATP binding cassette (ABC) superfamily, multi-drug and toxic-compound extrusion (MATE) family and the resistance nodulation division (RND) family are the five families of efflux pump systems. Biofilm formation combined with the presence of efflux pumps, reduces bacterial antibiotic sensitivity [7-9].

So far, there are few epidemiological studies on the frequency of antibiotic resistance patterns, biofilm formation and efflux pumps frequency in UTI-causing *E. coli* isolates in Iraq. Hence, this study aimed to look into the antibiotic resistance, biofilm formation and frequency of 5 major efflux pump families in UTI-causing E. coli isolates.

Materials and Methods

Ethics:

The present study was approved on 05.15.05.2020 by the Local Committee of the University of Babylon, Babylon, Hilla City, Iraq following the Declaration of Helsinki. All participants gave verbal consent to use their samples for research experiments.

Bacterial Isolates Identification:

Fifty *E. coli* were collected from a cystitis patients (350 patients, 150 male (21-65years) and 200 female (18-50 years). First the screening was done on MacConkey and eosin methylene blue (EMB) agar (Merck, Germany). Confirmation of *E. coli* was done via polymerase chain reaction (PCR) by means of primer pairs of *uidA* gene: forward primer: [TGGTAATTACCGACGAAAACGGC], reverse primer: [ACGCGTGGTTACAGTCTTGCG] to give 162 bp product at annealing temperature 60.9℃ [10].

Antibiotic Susceptibility Test: Antibiotic susceptibility test was performed against 26 antibiotics according to the clinical and laboratory standards institute (CLSI) by the disc-

diffusion assay. Isolates were activated for 18 hours at a temperature of 37°C in the nutrient broth. It was later adjusted to 0.5 Mc Farland's standard and spreading it on Mueller Hinton agar (MHA) by a swab. Antibiotic discs were positioned on MHA and lightly pushed down for ensuring whole contact through the bacteria-inoculated agar. They were then incubated for 18–20 hours at a 37°C and then the inhibition zone diameter (mm) was measured [11].

DNA Extraction and PCR for Detection of uidA:

The G-Spin Genomic DNA Extraction Kit (for bacteria) (iNtRON Bio Co, Korea) was used to separate genomic DNA from *E. coli* isolates. Using specific primer pairs, traditional PCR was utilized for amplifying 19 efflux pump genes (Table 1). The PCR conditions are illustrated in Table 2. The PCR mixture (20µl) consisted of 5µl of Maxime PCR Premix kit (i-Taq) (iNtRON Bio Co, Korea), 2.5µl of forward primer (10pmole/µl), 2.5µl of reverse primer (10pmole/µl), 5µl of target DNA, and 5µl of nuclease-free water (New Biolabs, US) [12].

Biofilm Formation:

As previously described, a 96-well polystyrene microtiter plate was used to evaluate biofilm formation by *E. coli.* Crystal violet (0.1%) was used to stain the biofilms for 30 minutes. Using 100% ethanol for 30 min, a crystal violet dye associated with biofilms was eluted, and its absorbance was measured at 595nm using tissue culture plate (TCP) assay described by Christensen *et al*[13].

Table 1 Oligonucleotide sequences and polymerase chain reaction conditions.

Efflux pump Class	Gene	Sequence	Product size bp	Annealing Temperature	Reference
RND	AcrA-F	ATCACCTTTCGCACTGTCGT	256	58.3ºC	This Study
	AcrA-R	CGACAAACAGGCCCAACAAG			
	AcrB-F	CATAAACACGCCCTGGTCCT	432	60.3ºC	
	AcrB-R	GCTACCCGTAAGTCGATGGG			
	AcrF-F	ATCCTCGCCGCTTTTGGTTA	626	57.2ºC	
	AcrF-R	AACACTTTTTGCGTCCGCTC			
	AcrE-F	CGCTGCAATTCTCCGATGTG	376	60.3ºC	
	AcrE-R	GCAGTATCTGCGGGGGTATC			
	AcrD-F	GCCGTGCAGCAAGTACAAAA	424	58.3ºC	
	AcrD-R	CTGGTGTTTGCAGCAGTGAC			
MFS	mdfA-F	TTCGATGACCGCGTATCTGG	206	59.3ºC	
	mdfA-R	CAGCGCCAATGAAACAGAGG			
	EmrD-F	ACGTTAATGTGGCAGTCGGT	233	57.2ºC	
	EmrD-R	ACGCCGGAACCAATGTTTTG			
	emrA-F	CGCTGGAGCGTACTCGTATT	287	58.3ºC	
	emrA-R	ATTTTGCGCTGGAAGCAGTG			
	emrB-F	CTGCGCCGGTAGGGATTATT	436	59.3ºC	
	emrB-R	ATCCCAAGCCCTTCCAGTTG			
SMR	EmrE-F	ACACGGTTATGGCCATCTGT	249	57.2ºC	
	EmrE-R	ATGTGGTGTGCTTCGTGACA			
	YnfA-F	CGCACCCGTCCAGTCATAAA	220	58.3ºC	
	YnfA-F	ATGCTTTCTGCCCTGGTTGT			
	TehA-F	TTGCCCGACTCATACGCTTT	208	58.3ºC	
	TehA-R	CGCCATAATCCCGCAGTTTG			
ABC	MacA-F	GCACAACAAGCACCGAACAT	255	58.3ºC	
	MacA-R	CATATCCAGCCGCAGCAAAC			
	MacB-F	GCGTGAGCGGCGATTATTTT	364	57.2ºC	

	MacB-R	AAACGCGTGAGTTGCTGTTC		
	MdlA-F	GCGCGTATTGATGCTCGTTT	383	58.3ºC
	MdlA-R	AGCATCTGACCGGGTTTCAG		
	MdlB-F	GTTACGCCAGCCATTAAGCG	227	59.3ºC
	MdlB-R	TCACCATTACCACCAGCACC		
	MdtK-F	CTCTTTGGTCACGGACTGCT	350	59.3ºC
MATE	MdtK-R	TTCACCGGGATGTTCACCAG		
	DinF-F	TTGGTCTGCTAATGGTGCGT	400	58.3ºC
	DinF-R	ATGATGTGTTCCCCAGCCAG		
	TolC-F	CGATCGTGATGCTGCCTTTG	596	58.3ºC
	TolC-R	GGTTGCGTTTTTCGGCTTCT		

Results:

Antibiotic Susceptibility:

The results revealed that all isolates have been resistant to amoxicillin, ceftazidime and cefotaxime, while all isolates were sensitive to imipenem (Figure 1). Less resistance was applied to levofloxacin (12%), azithromycin (10%), doxycycline (8%), meropenem (6%), gentamicin (4%)and netilmicin (2%).

Antibiotics Resistance Patterns:

Concerned antibiotic resistance patterns MDR (resistance to at least single antibiotic from 3 different classes), XDR (resistance to at least single antibiotic from all antibiotic classes except two) and PDR (resistance to at least single antibiotic from all antibiotic classes), the results revealed that 19 (38%) *E. coli* isolates were non-MDR while 31 (62%) were MDR with variable number of classes resistance: 2 (4%), 6 (12%), 1 (2%), 12 (24%), 10 (20%) for MDR-7 classes, 6 classes, 5 classes, 4 classes, and 3 classes, respectively (Figure 2).

Coexisted Resistance Patterns for UPEC:

The highest coexisted resistance phenotypes for *E. coli* distributed as 8 (16%) for betalactam/aminoglycosides resistance and 5 (10%) for betalactam/aminoglycosides/nitrofuran resistance (Table 2).

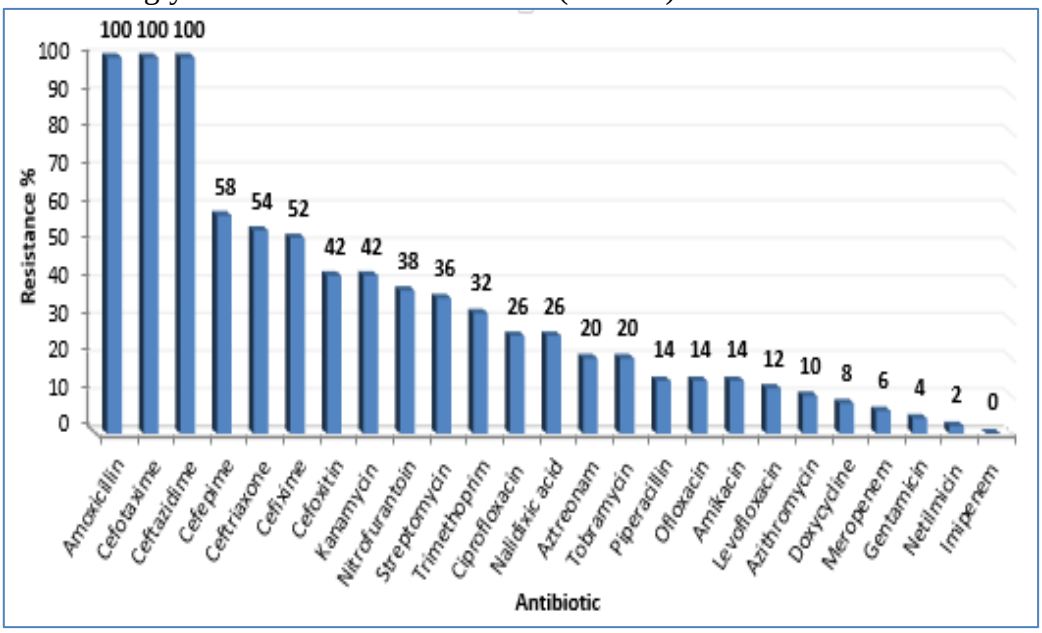

Figure 1 - Antibiotic resistance among *Escherichia coli* isolates.

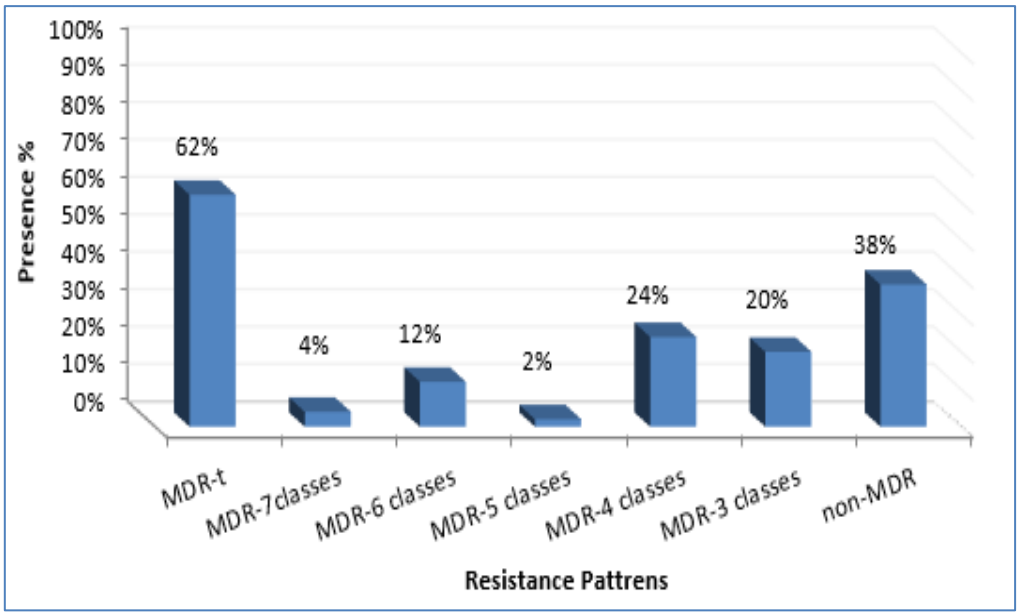

Figure 2 - Resistance patterns among *Escherichia coli* isolates.

Table 2 Phenotype of coexisted antibiotic resistance among *Escherichia coli* isolates.

Classes	Phenotypes	Resistance Pattern	NO.	%
7	B-Lactam/FLOURO/CARBA/MONO/AMINO/NITRO/MACRO	MDR	1	2
	B-Lactam/FLOURO/AMINO/TETRA/SULFA/NITRO/MACRO	MDR	1	2
6	B-Lactam/FLOURO/MONO/AMINO/TETRA/NITRO	MDR	1	2
	B-Lactam/FLOURO/MONO/AMINO/TETRA/SULFA	MDR	1	2
	B-Lactam/FLOURO/CARBA/MONO/AMINO/SULFA	MDR	2	4
	B-Lactam/FLOURO/MONO/AMINO/SULFA/MACRO	MDR	1	2
	B-Lactam/FLOURO/MONO/AMINO/SULFA/NITRO	MDR	1	2
5	B-Lactam/FLOURO/AMINO/TETRA/SULFA	MDR	1	2
	B-Lactam/AMINO/NITRO/MACRO	MDR	1	2
	B-Lactam/FLOURO/AMINO/NITRO	MDR	1	2
	B-Lactam/CARBA/AMINO/MACRO	MDR	1	2
	B-Lactam/CARBA/AMINO/NITRO	MDR	1	2
4	B-Lactam/FLOURO/MONO/AMINO	MDR	1	2
	B-Lactam/FLOURO/AMINO/SULFA	MDR	1	2
	B-Lactam/AMINO/SULFA/NITRO	MDR	3	6
	B-Lactam/MONO/AMINO/NITRO	MDR	2	4
	B-Lactam/FLOURO/AMINO/NITRO	MDR	1	2
	B-Lactam/AMINO/SULFA	MDR	2	4
	B-Lactam/FLOURO/AMINO	MDR	1	2
3	B-Lactam/AMINO/NITRO	MDR	5	10
	B-Lactam/SULFA/NITRO	MDR	1	2
	B-Lactam/AMINO/SULFA	MDR	1	2
	B-Lactam/AMINO	MDR	8	16
2	B-Lactam/FLOURO	non-MDR	1	2
	B-Lactam/Tetracyclin	non-MDR	1	2
1	B-Lactam	non-MDR	9	18
Total			50	100

Molecular Detection of Efflux Pumps for UPEC:

Results of molecular investigation of efflux pumps in *E. coli* revealed that, class RND AcrAB-TolC, AcrAD-TolC and AcrFE-TolC genes were distributed as following: *acrA* 50 (100%), *acrB* 43 (86%), *acrD* 48 (96%), *acrF* 33(66%), *acrE* 50 (100%) and *tolC* 50(100%). Three class MFS pumps (EmrAB-TolC, EmrD and MdfA) were also investigated for *E. coli* and the results were as following: *emrA* 50 (100%), *emrB* 50 (100%), *emrD* 50(100%) and *mdfA* 49 (98%). Three class SMR pumps (EmrE, YnfA and TehA) genes were distributed as follows: *emrE* 48 (96%), *ynfA* 50 (100%) and *tehA* 49(98%). Two class ABC pumps (MacAB-TolC and MdlAB-TolC) genes distribution were as following: *macA* 50 (100%), *macB* 49 (98%), *mdlA* 50 (100%) and *mdlB* 49 (98%). Two MATE pumps MdtK and DinF were detected in all *E. coli* isolates.

Coexisted Genotypes of Efflux Pumps for UPEC:

Concern results of coexisted pumps in same *E. coli* isolates showed that 32 (64%) of isolates have genotype AcrAB-TolC/ AcrAD-TolC/ AcrFE-TolC/ MdfA/ EmrD/ EmrAB-TolC/ EmrE/ YnfA/ TehA/ MacAB-TolC/ MdlAB-TolC/ Mdtk/ DinF, while 16 (32%) have genotype AcrAB-TolC/ AcrAD-TolC/ MdfA/ EmrD/ EmrAB-TolC/ EmrE/ YnfA/ TehA/ MacAB-TolC/ MdlAB-TolC/ Mdtk/ DinF (Table 3).

Table 3-Genotypes of different efflux pumps among *Escherichia coli* isolates.

Genotype	No.	%
AcrAB-TolC/ AcrAD-TolC/ AcrFE-TolC/ MdfA/ EmrD/ EmrAB-TolC/ EmrE/ YnfA/ TehA/ MacAB-TolC/ MdlAB-TolC/ Mdtk/ DinF	32	64
AcrAB-TolC/ AcrAD-TolC/ AcrFE-TolC/ MdfA/ EmrD/ EmrAB-TolC/ YnfA/ TehA/ MacAB-TolC/ MdlAB-TolC/ Mdtk/ DinF	1	2
AcrAB-TolC/ AcrAD-TolC/ MdfA/ EmrD/ EmrAB-TolC/ EmrE/ YnfA/ TehA/ MacAB-TolC/ MdlAB-TolC/ Mdtk/ DinF	16	32
AcrAB-TolC/ AcrAD-TolC/ MdfA/ EmrD/ EmrAB-TolC/ EmrE/ YnfA/ MacAB-TolC/ MdlAB-TolC/ Mdtk/ DinF	1	2

Biofilm Formation for UPEC:

Results of biofilm construction showed that 98% of bacterial isolates were biofilm former while 2% were non-biofilm. In total, 60% were weak biofilm producers, 24% were moderate, and 14% strong biofilm formers (Table 4).

Table 4-Biofilm formation patterns among *Escherichia coli* isolates.

Biofilm formation pattern	No.	%
Weak biofilm former	30	60
Moderate biofilm former	12	24
Strong biofilm former	7	14
Non Biofilm former	1	2

Discussion:

Because of the high recurrence rate and widespread antibiotic resistance, UTIs are a major public health concern. Various researchers have found a high prevalence of antibiotic resistance amongst the UPEC isolates, with MDR phenotype [14-16]. In this study, antibiotic resistance testing indicated complete resistance to cefotaxime, amoxicillin and ceftazidime, which could be due to the efflux pump. There is a strong link between the existence of 5 classes of efflux pump genes and their resistance to those three antibiotics. Multidrug efflux pumps are involved in antibiotic resistance in a variety of ways [17, 18]. In *E. coli* clinical isolates, AcrAB-TolC pump, which belongs to RND family, represents the major efflux pump [19, 20].

Our findings are consistent with various other researchers, such as those of Mahmoud and Rizk (2018), who discovered that the existence of *TolC* and *acrA/B* genes in *E. coli* were considerably related to antibiotic resistance [20]. The *acrB*, *acrA*, and *tolC* genes have been identified in 74.84% of MDR-UPEC isolates, according to Elsayed *et al*. [21]. In 68.5% of cases, all 3 genes *acrB*, *acrA*, and *tolC* exist at the same time [22]. Even – with low fluoroquinolone resistance, AcrAB-TolC might be involved in such resistance, based on the records from Iranian research that found *acrB* and *acrA* genes in all ciprofloxacin-resistant *E.coli* [23, 24]. Overexpression of the *mdfA* and *acAB-tolC* genes may contribute to fluoroquinolone resistance [25, 26]. All carbapenem resistant *E.coli* isolates had more overexpression of AcrAb-TolC [27]. Another research found a link between the existence of AcrAB-TolC genes and nalidixic acid resistance in the form of ofloxacin [28]. Russian research suggests that AcrAb-TolC pump is involved in the long-term cleaning of many abiotic and biotic agents [29].

AcrAD-TolC, a transporter from the RND family was found to participate in aminoglycoside efflux. The MICs of gentamicin, amikacin, kanamycin, neomycin and tobramycin were reduced when the *acrD* gene was deleted [30]. *AcrD* might also extrude aminoglycosides into the medium by capturing them in periplasm [31]. TolC is required for the functions regarding all RND drug transporter systems in *E. coli* (AcrEF, AcrD, and MdtA) [32, 33]. AcrEF-TolC is of high importance in fluoroquinolone resistance, according to research from China and India [34, 35,36]. In a single isolate, efflux pump-mediated resistance to multiple antibiotics was found in 95% of isolates, all of which have been MDR UPEC [17]. EmrAB–TolC is a tripartite system made up of EmrB, an MFS transporter, EmrA, a membrane fusion protein and TolC, an outer membrane channel from *E. coli*.

MacB can be defined as one of the ABC-type macrolide efflux transporters that work in tandem with MacA, the MFP, and TolC, the multi-functional outer membrane channel [37, 38]. In macrolide-susceptible AcrAB-deficient *E. coli*, overproduction of MacAB leads to increased resistance to macrolide antibiotics [39]. In *E. coli*, ynfA, a member of the SMR efflux pump gene family, increases antibiotic resistance. Antibiotic resistance to penicillins and cephalosporins is also thought to develop in ynfA-expressing *E. coli* [40, 41]. The findings showed a strong link between biofilm formation and the presence of efflux pump genes.Evidences suggest that pumps play an important role in the formation of biofilms [42]. The emergence of MDR and XDR bacteria in recent years has made epidemiological studies of special importance in finding out exactly how these resistant bacteria work [43-48].

Conclusion:

The current study concluded that all efflux pumps may be highly associated with resistance to amoxicillin, cefotaxime, and ceftazidime and moderately associated with cefepime, ceftriaxone and Cefixime. These efflux pumps may be unrelated to the resistance of other studied antibiotics or the perhaps concentration of these antibiotics could be inadequate to induce the expression of these pumps. Additionally, biofilm formation was found to be highly related to the presence of study pumps.

Conflict of Interest:

The authors declared that there is no conflict of interest

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