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Department of Biology – College of Science – University of Diyala, Iraq.

wedad.adil.kadhim@gmail.com

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Abstract

A total of 87 clinical specimens from midstream urine samples were collected from children with urinary tract infection, all specimens were inoculated on suitable media for isolation and primary identification of bacteria then biochemical tests were performed, the final diagnosis using the VITEK-2 system, 25(28.7%) isolates of *Escherichia coli* were obtained. Antimicrobial susceptibility test of isolates showed that *E*scherichia *coli* isolates were highly resistance to most antibiotics used and presence of multidrug resistant. The prevalence of, efflux pumps were 60%. Current study of gene detection of efflux pumps genes reveal presence of *acrA* and *acrB* genes in all isolates (100%), treatment of isolates with sub –MIC Ciprofloxacin gene show inhibition of expression of the *acrA* and *acrB* genes.

Keywords: Escherichia coli, efflux pumps, acrA gene, acrB gene

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الكشف عن التعبير الجيني لمضخات الدفق في الاشيرشيا القولونية المعزولة من التهاب المسالك البولية عند الاطفال

وداد عادل كاظم & الاستاذ الدكتور كريم ابراهيم مبارك

قسم علوم الحياة ، كلية العلوم ، جامعة ديالى ، العراق.

الخلاصة:

تم جمع 87 عينة سريرية من عينات البول في منتصف الجريان من مرضى الأطفال المصابين بعدوى المسالك البولية ، وتم زرع جميع العينات على وسط مناسب لعزلها ،وتم تأكيد تشخيصها بوساطة الاختبارات البيوكيميائية ، ونظام . VITEK-2 حيث تم الحصول على (28.7%)25 عزلة من الاشيرشيا القولونية. أظهر اختبار الحساسية للمضادات الحياة أن عزلات *Escherichia.coli* كانت عالية المقاومة لمعظم المضادات الحياة المستخدمة ووجود مقاومة متعددة للأدوية. بلغت نسبة انتشار مضخات الدفق 60٪. أظهرت الدراسة الحالية للكشف الجزيئي عن جينات مضخات الدفق عن وجود جينات *acrA* و *acrB* في جميع العزلات (100٪) ، كما أظهرت الدراسة تثبيط التعبير الجيني لجينات *acrA,acrB* بعد معاملة العزلات البكتيرية ب Sub-MIC Ciprofloxacin.

الكلمات المفتاحية:

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Introduction

Urinary tract infection (UTI) is considered one of bacterial disease with a high morbidity with word wide distribution [1]**.** In the first year of life particularly in those with anatomical or functional abnormalities show high distribution urinary tract infections [2]. *Escherichia coli* the most commonly encountered bacteria that cause (UTI) in addition to other gram negative species include Citrobacter spp., *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, and *Proteus vulgaris* [3]. *Escherichia coli* is a rod-shaped gram-negative bacteria that is facultative anaerobic and lactose fermentative [4]. It is one of the most prevalent forms of Enterobacteriaceae bacteria found in the human gut, making it capable of infecting other body systems when the opportunity arises and causing a variety of disorders [5]. *E. coli* is the leading cause urinary tract infections in both men and women, and it ranks second only to respiratory disorders. [*6*]. Due to bacteria's ability to excretion several enzymes, such as [ESBL] Extended Spectrum B-lactamase Enzymes [ESBL], metallo B-lactamase [MBL], and hemolysin.

Excessive use of antimicrobial drugs has resulted in challenging treatment of UTI infection [7]. Antibiotic resistance is conferred on the bacterium due to many factors include the permeability of the cellular membrane, changing the target site, efflux pumps, suppressing protein synthesis, and others [*8*].

Efflux pumps are transporters protein found in bacteria's cell membranes that play a key function in transporting various compounds and expelling them outside the cell to reduce their damaging effects They are also a key mechanism by which bacteria develop antibiotic resistance [9]. Chromosomal efflux pumps, which carry the genes that code for them on a cell's chromosome, and plasmid efflux pumps, which carry the genes that encode for them on portable genetic materials like plasmids or transposons, are the two basic types of efflux pumps [10]. Major Facilitator Super Family (MFS), Family [Multidrug and Toxic Efflux Family (MATE),

ATP-Binding Cassette Family (ABC), Small Multidug Resistance Family (SMR), Resistance - Nodulation - Division Family (RND) are the five types of efflux pump families [11]. The RND family is divided into three categories depending on its components: Single-component efflux pumps, which transport hydrophilic antigens and are represented by the protein AcrB found in the cell's inner membrane and encoded by the *acrB* gene. AcrB in the inner membrane and AcrA lipoproteins in the periplasmic space are two-component pumps that are encoded by the *acrA* gene. AcrB in the inner membrane, AcrA proteins in the plasma vacuole, and a funnel-like protein channel termed ToIC produced by the *tolC* gene in the bacterium's outer membrane make up the three-component tripartite pumps [*12*].

Gene expression has long been recognized as a necessary component of cell function. It takes many forms and is organized in both positive and negative ways. When mRNA is first transcribed in prokaryotic cells, regulation occurs. While it becomes more complex in eukaryotes, there are multiple mechanisms to complete the regulatory process. This process occurs through the association of proteins with a specific sequence on the DNA strand, resulting in an increase or decrease in the transcription rate in both eukaryotic and prokaryotic cells. Cloning initiates the process in bacteria. The lactose operon system, for example, is under both positive and negative control [13].

The high antibiotics resistance among the most common uropathogenic *Escherichia coli* as etiological agent of UTI, and the important of the efflux pumps in the resistance, the current study were conducted to determine the MDR isolates from UTI in children in Baquba city, the prevalence of *acrA* and *acrB* genes and the effect of SUB –MIC Ciprofloxacin treatment on gene expression of bacterium isolates.

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Materials And Methods

Collection of urine Specimens

Eighty seven mid stream urine specimens were collected from children with signs of urinary tract infection admitted to Al-Batool Maternity and Children Teaching Hospital in Baquba city during the period of 1/9/2021 to 1/12/2021, by using disposable collection sterilize container. The inoculation of each specimens done direct, the information about each patient recorded .

Isolation and identification of bacteria

The collected urine specimens were inoculated directly on the, MacConkey agar medium, incubated aerobically overnight at 37C, the expected isolates were subculture on the Eosine Methylene Blue (EMB) agar and re incubated in the same conditions. identification performed according to morphological (grams stain, shape of cells and colony characters), biochemical testes including (oxidase, catalase IMVIC tests, urease, Triple sugar iron), and the confirmation of bacterium identification done by used the VITEK-2 compact system [*14*].

Antibiotic sensitivity test and MIC Determination

the susceptibility of *E.coli* isolates to antibiotics were performed by use 14 antibiotics of (gentamicin , amikacin, norfloxacin, ciprofloxacin, ofloxacin, trimethoprim ,chloramphenicol, colistin sulfate, tetracyclin, azithromycin, cefoxitin , aztreonam, amoxicillin-clavulanate , ampicillin Dose). According use the base of the Disk Diffusion method by spreading bacteria on the Muller-Hinton Agar medium [15]. the interpretation of results and classify isolates as sensitive and resistance by (CLSI 2019). Determination of MIC of (Ciprofloxacin & Norfloxacin) [*16*].

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Phenotypic Efflux pumps detection

Phenotypic Detection of Efflux pumps were conucted by (cartwheel pattern) agar-EtBr method [17], dilutions of bacterial were prepared from isolates that reveal the resistance to antibiotics and has the ability to biofilm formation after incubated for 24 hours at 24C in the 5 ml of buffer and their concentration were adjusted by compare to 0.5 of MacFarLand standerd solution , then isolates inoculated on tryptic soy agar (TSA) plates containing (0,5,10,15,20,25 µg\ml) of Ethidium Bromide (EB) ,the plate were divided to 16 by radial lines (cartwheel pattern) and the plates incubated for 16 hours at 37 C in the dark place then the isolates were examined under ultra violet (UV) trans illuminator to record the fluorescent.

Genotypic detection of efflux pumps:

DNA extraction: DNA bacterial was extracted from the isolates of *E.coli* by using genomic DNA purification Kit supplemented by manufactured company (Geneaid, Thailand). and DNA was purified and their purity was measured by using nano-drop spectrophotometer [*18*].

Polymerase Chain Reaction (PCR): Used to detected *acrAB* efflux pump genes (*acrA*, *acrB*) in *E.coli* isolates. Proliferation of *acrAB* genes with two primer pairs (Bioneer, korea) table 1. The amplification of DNA was performed at A volume of 25µl, the reaction mixture was containd of 5µl Accu powerPCRPremix (Bioneer, korea), 3µl template DNA, 1.5µl F-primer, 1.5µl R-primer and 14µl deionized nuclease free water. PCR reaction of *acrAB* genes were performed according to [*11*] in three steps: first initial DNA denaturation at 95C for 5 minutes (1 cycle), denaturation of DNA template at 95C for 30 seconds to amplify DNA, annealing at 52C for 30 seconds and extension at 72C for 45 seconds (30 cycles) and final extension at 72C for 5 minutes (1 cycle).

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Table 1: Sequences and primers concentration and used in the study

Gen	The primer sequence [5’-3[’	Output size bp	Reference	Tm [C[̊
acrA	F-CTCTCAGGCAGCTTAGCCCTAA R-TGCAGAGGTTCAGTTTTGACTTT	107	Maleki *et al* [2017[	52
acrB	F-GGTCGATTCCGTTCTCCGTTA R-CTACCTGGAAGTAAACGTCATTGGT	105	Maleki *et al* [2017[	52

Gel electrophoresis: after the amplification process in thermo cycler PCR apparatus, the product were run on 1.5 % agarose gel with 5µl of ethidium bromide in 1x (TBE) buffer using DNA ladder (100-2000) bp (Bioneer, Korea) at 100 volte for 80 minutes. The visualization of PCR products under 320 nm UV light by using of a UV transilluminator [29]

Ciprofloxacin Effect on gene expression

RNA Isolation: E.coli Bacteria was pelleted then lysed in 1 ml of AccuZolTM lysate through the, two hundred µl of chloroform was added per 1ml of AccuZolTM and shake vigorously for 15 seconds, The mixture was incubated on ice for 5 minutes, the mixture was centrifuged at 12000 rpm for 15 minutes at 4C, the aqueous phase was transferred to a new 1.5ml tube and equal volume of isopropyl alcohol was added and the tubes were mixed by inverting 4 – 5 times and it incubated at -20C for 10 minutes then Centrifuge at 12000 rpm for 10 minutes at 4C was done, then supernatant carefully was removed One ml of 80% ethanol was added and mixed well by vortexing and Centrifuge at 12000 rpm for 5 minutes at 4C was done, then supernatant was carefully remove and the pellet was dried, the RNA was dissolved in RNase – free water by passing the solution a few times through the pipettes tip and stored at -80% until us [20].

Transformation RNA to DNA: the RNA extracted from bacterial isolate, primer and water were mixed and incubated at 65C for 5 minutes, on ice for two minutes then RNA were 10mg-500mg, random primer 1µl, 2XES reaction mix 10µl, RT\RL enzyme 1µl, gDNA remover 1µl,

RNase- free water 20µl (Bioneer, korea) the mixture was treated with 25C for 10 minutes,42C for 15 minute and 85C for 5 second to intact enzymes.

Real-time quantitive reverse transcription [qRT-PCR]: RT-qPCR is performed in a reaction solution of 20µL volume using BrightGreen qPCR MasterMix by mixing the 0.6 µL of primer, 10µL of BrightGreen 2X qPCR mastermix, ≤ 500 ng of cDNA, Nuclease - free H2O to 20 µl.

The generated solution was placed in Real time PCR Cycler for thermal reaction to measure the Cycle Threshold [CT] value. RT-PCR is used for quantification of the levels of gene expression. The measured CT values during thermal reaction are recorded to computer the following measurements [21].

Real Time qRT-PCR analysis: ΔCT (test) = CT gene of interest (target, test) – CT internal control, ΔΔ CT=ΔCT (test)- ΔCT (calibrator), $2^{-\Delta\Delta Ct}$ = Normalized expression ratio [22].

Statistical analysis

Statistical analysis was carried out using the SPSS version 20 and the results were analyzed using the Chi Square test with a probability level of P≤ 0.05 [23].

Results And Discussion

The results of the diagnosis showed that the number of *E.coli* isolates obtaind from a total of 87 urine specimens were 25 (28.7 %). Biochemical tests reveal that all the isolates of *E.coli* were positive for catalase, Indole, Methy Red and negative for Oxidase, Vogues-proskauer , citrate utilization and Urease, TSI (A\A,H2S+, gas+). *E.coli* isolates were gave pink colonies on MacConkey agar, dark blue with green metallic sheen on (EMB) agar observed that *E.coli* (68 .9 %) where the predominant microorganism among Gram-negative bacteria were the main cause of UTIs [14].

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Sensitivity of *E.coli* to antibiotics:

The results of the current study showed the resistance ratio to Gentamicin is 2 [8%]. this result were do not agree to [24], which showed that the resistance to the Gentamicin is 15%, while the resistance ratio *E.coli* isolates to Amikacin was 1(4%).

This result was do not agree to [25], which showed that the resistance to the Amikacin is 0%. while the resistance ratio *E.coli* isolates to norfloxacin was 11 (44%) this result was do not agree to [25]. while the resistance ratio *E.coli* isolates to norfloxacin was11 (44%) this result was do not agree to [25]. which showed that the resistance to the antibiotic 81.7%.

The resistance ratio *E.coli* isolates to ciprofloxacin is 9 (36%). This result were do not agree to [24]. which showed that the resistance to the antibiotic 51%. while the resistance ratio *E.coli* isolates to Aztreonam and Cefoxitin were 8 (32%) this results were do not agree to [24]. which showed that the resistance to the antibiotics were (60%, 35%). While the resistance ratio *E.coli* isolates to Trimethoprim 4 (%16). This result was approached to [24].

The resistance ratio *E.coli* isolates to Amoxicillin 21 (84%) and Ampicillin 22 [88%]. This study do not agree to [24]. Which showed that the resistance to the antibiotics 92% table 2.

Table 2: Resistant Limit for antibiotics to *E. coli* bacteria

Antibiotic	Resistant	Intermediate	Sensitive

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Antibiotic	Group	no	%	no	%	No	%
Gentamicin	Aminoglycoside	2	8	5	20	18	72
Amikacin		1	4	1	4	23	92
Norfloxacin	Quinolones& Fluoroquindones	11	44	2	8	12	48
Ciprofloxacin		9	36	2	8	14	56
Ofloxacin		7	28	5	20	13	52
Trimethoprim	Folate pathway Antiggonists	4	16	2	8	19	76
Chloramphenicol	Phenicol	20	80	2	8	3	12
Colistin sulfate	Lipopiptide	4	16	11	44	10	40
Azithromycin	Macrolidees	12	48	4	16	9	36
Aztreonam	Monocyclin	8	32	14	46	3	12
Cefoxitin	Cephalosporins	8	32	10	40	7	28
Amoxicillin-Clavulanate	B-lactam combination	21	84	3	12	1	4
Ampicillin	Penecilins	22	88	3	12	0	0

MIC Determination: The current study revealed a contrast in the MIC values of *E.coli* isolates against the antibiotics ciprofloxacin and norfloxacin for isolates of children infected with urinary tract infections. Where it ranged between (4-512) µg/ml for ciprofloxacin, and (4-1024) µg/ml for norfloxacin **and** the rang of MIC for the four isolates [16, 29, 50,49] were that treated to study the gene expression reach (4-128) µg\ml.

Efflux pumps production: The results of the current study showed that 15 (60%) of *E.coli* produce efflux pumps. This result do not agree to [26], who found that 70% of *E.coli* isolates are efflux pumps producers.

Detection of (*acrAB*)genes: molecular detection indicate that all isolates were carrying *acrAB* genes figures (1 and 2) the results agreed with the results of [26].

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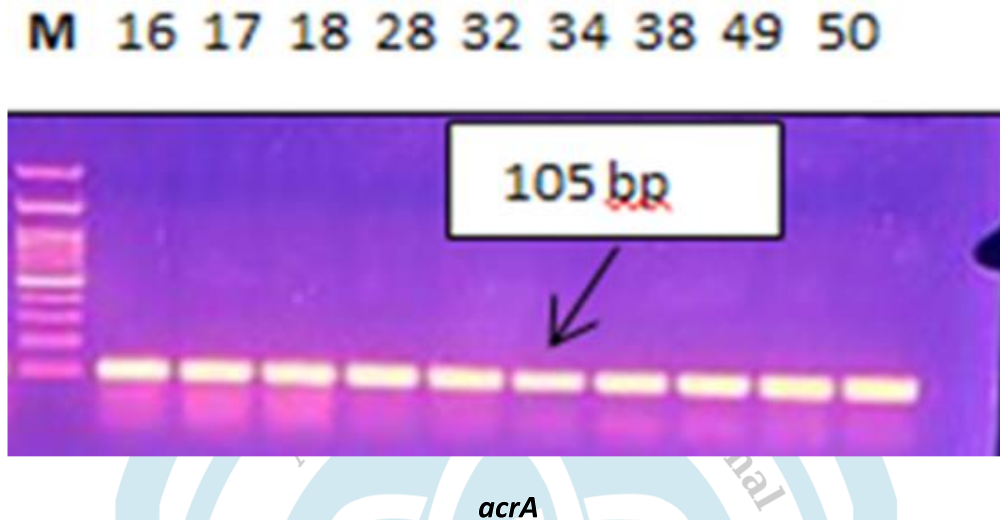

Figure 1: Electrophoresis of amplified *acrA* (105bp) for *E.coli* by 1.5% agarose gel, using DNA Ladder 100-2000bp, 80V\cm for 80 min, staind with ethidium bromid dye and visualized under UV transilluminator documentation All lanes are shown to be have the gene.

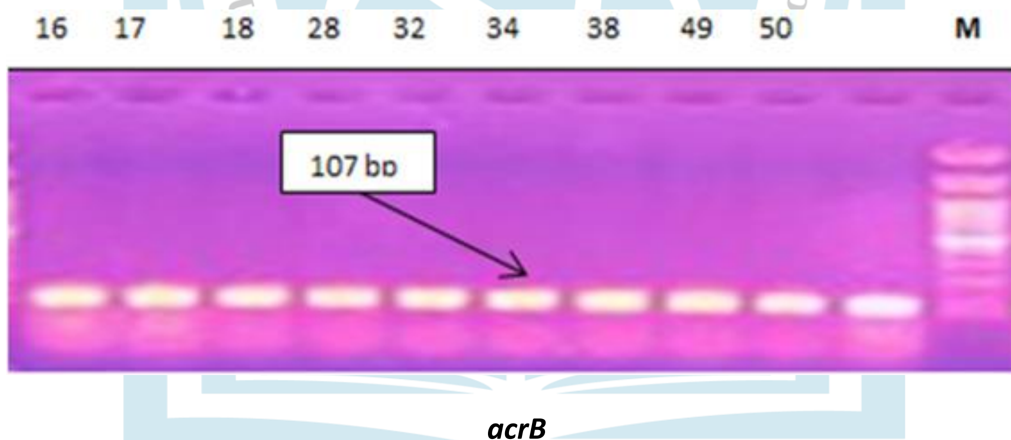

Figure 2: Electrophoresis of amplified *acrB* (107bp) for *E.coli* by 1.5% agarose gel, using DNA Ladder 100-2000bp, 80V\cm for 80 min, staind with ethidium bromid dye and visualized under UV transilluminator documentation All lanes are shown to be have the gene.

Ciprofloxacin Effect on gene expression: The results of the current study for four bacterial *E.coli* of isolates (16, 29, 50,49) isolated from children before and after treatment with

Ciprofloxacin using qRT-PCR technique. using the primers described in table [1]. showed an amplification of the *acrA* gene with a value of threshold cycle (CT) before isolates (16, 29, 50,49) treated with sub MIC CIP, Where recorded (31.96, 32.11, 28.17, 29.27). the quantitative changes in mRNA were determined using an equation Threshold limit Ct ($2^{-\triangle\triangle ct}$), as shown in Table [3]. After treatment of the isolates with CIP. Decreased gene expression after treatment with cip, table 3.

Table 3: effect of ciprofloxacin treatment on CT and Fold values of *acrA* gene expression for *E.coli*

ANTIBIOTIC	NO OF ISOLATE	MEAN] H.K.[	MEAN *ACRA*	DCT	DDCT	FOLDING	P. VALUE
Ciprofloxacin after treatment sub –Mic	16	24.83	34.87	10.04	0.11	0.9265	*0.05
	29	22.77	34.57	11.8	-0.17	1.1250	
	50	23.35	29.05	5.7	0.55	0.6830	
	49	22.85	30.01	7.16	0.21	0.8645	
Control before treatment sub –Mic	16	22.03	31.96	9.93	0	1	0.00
	29	20.14	32.11	11.97	0	1	
	50	23.02	28.17	5.15	0	1	
	49	22.32	29.27	6.95	0	1	

results of the current study for four bacterial *E.coli* of isolates (16, 29, 50,49) isolated from children before and after treatment with Ciprofloxacin using qRT-PCR technique. using the primers described in table 1 showed an amplification of the *acrB* gene with a value of threshold cycle (CT) before isolates (16,29 ,50 ,49) treated with sub MIC CIP, Where recorded (23.39, 24.91, 27.02, 28.02) the quantitative changes in mRNA were determined using an equation Threshold limit Ct($2^{-\triangle\triangle ct}$), as shown in table 4. After treatment of the isolates with CIP. Decreased gene expression after treatment with cip table 4.

Our study not agee with the [27],which showed increased gene expression for *acrAB* after treatment with cip.

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Table 4: Effect of ciprofloxacin treatment on CT and Fold values of *acrB* gene expression for *E.coli*

Antibiotic ug /ml	No of isolate	Mean (H.K.)	Mean *acrB*	dCT	ddCT	folding	p.value
ciprofloxacin after treatment sub -Mic	16	24.83	37.14	12.31	10.95	0.0005	*0.006
	29	25.32	35.02	9.7	7.21	0.0067	
	50	23.35	25.35	2.28	0.13	0.91383	
	49	20.98	28.91	7.93	0.03	0.9794	
Control Before treatment Sub /Mic	16	22.03	23.39	1.36	0	1	0.00
	29	22.42	24.91	2.49	0	1	
	50	24.87	27.02	2.15	0	1	
	49	20.12	28.02	7.9	0	1	

Conclusion

The results of isolation and diagnosis of *Escherichia coli* bacteria showed multi-resistance to antibiotics due to their possession of efflux pumps.The results of the gene expression of *acrA* and *acrB* genes were decreased gene expression, after treatment with ciprofloxacin antibiotic, due to agenetic mutation as a physiological response to the presence of an antibiotic.

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