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Correlation of *oprD* gene expression with *mexA* and *mexB* efflux pump gene Expressions under ciprofloxacin stress in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa, *mexA*, *mexB*, Cather Wheel.

ABSTRACT

Pseudomonas aeruginosa is an ubiquitous bacteria, has ability to adapt to different environments and causing in humans specially in Immunocompromised persons and hospitals. So the study aimed to detect the correlation between gene expressions of oprD and each of *mexA* and *mexB* genes in *Pseudomonas aeruginosa* isolates, fifty *P. aeruginosa* isolates were isolated from different clinical samples, detection of efflux pumps activity in *P. aeruginosa* phenotypically was done by Cather wheel method. Study genes expression of efflux pump genes under ciprofloxacin antibiotic stress and Et.Br. Results of *oprD* Ct values ranged from 20.56 to 24.98 with an average of 23.141. *mexA* Ct values were ranged from 32.1 to 36.79 with average 34.337 and results of *mexB* Ct values were ranged from 14.74 to 19.96 with average 16.791 compare to *rpsL* (Ct = 11.14) reference gene. The results showed that mean of Ct of gene expression was noticed when put them under stress of Et.Br. + CIP and CIP in *mexA* was 37.195 and 34.702, respectively, compare to control 34.337, on the other hand the gene expression between both treatments was over expressed in treatment of CIP 32 compare to CIP+Et.Br. The results of Cather wheel method expression was noticed when put them under stress Et.Br. + CIP and CIP in *mexB* gene expression was 19.626 and 18.872, respectively compare to control 16.791, In conclusion the both treatments compare to control not induce the expression of *mexB* genes, on the other hand the gene expression between both treatments was expressed in treatment of CIP 32 compare to CIP+Et.Br.

1. Introduction

Pseudomonas aeruginosa is an opportunistic bacteria, can adapt in different types of environments making them life-threating pathogens for human especially Immuno-compromised persons, moreover to their ability for causing diseases in hospitals [1]. multidrug-resistant (MDR) strains of *Pseudomonas* spp. became more critical especially with limited therapy considered, make infection with these bacteria big problem and may due to death [2], [3]. Many factors such as permeability of outer membrane, the efflux pumps

expressions , and the synthesis of antibiotic inactivating enzymes, all these reasons contribute to intrinsic resistance, especially the acquired resistance which may occurs as a result of mutational alterations or horizontal transfer of resistance genes by mobile genetic elements (MGEs) such as integrons, transposons, or plasmids, this acquired resistance leads to the emergence of new strains that are more resistant to antibiotics [3]. The ability of bacterial cell envelope to block the entry of antibiotics is referred to as the ″permeability barrier″, but it is becoming more and more obvious that the function of efflux pumps, whether acting on their own or in conjunction with decreased expression of porins, is a component of this ″barrier″ [4]. Limiting the quantity of medicine that may enter bacterial cell is therefore a crucial element. Efflux pump genes can be found on chromosomes or plasmids such as transmissible elements, any bacteria that can reproduce plasmids can acquire the genes for efflux pumps there in, however not all individuals of given bacterial species carry these genes [5]. Different mechanisms control the production of efflux pump; whereas some are perpetually production and give intrinsic tolerance or resistance to their substrates, others are only momentarily incited by substrates. When both forms of expression are enhanced either the inducer or a gene mutation that inhibits efflux pump expression causes increased efflux of the substrate. The membrane transporter proteins known as efflux pump (EPs) are crucial for *P. aeruginosa's* inherent and acquired antibiotics resistance mechanisms [6]. This organism's low outer membrane permeability and constitutive production of several EPs with broad substrate specificity make it naturally resistant to variety of structurally unrelated antimicrobial drugs [7], [8]. The most known systems of efflux are MexAB-DprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM, these systems are able to efflux number of antibiotics which found especially in *P. aeruginosa* due to multidrug resistance. Each pump is targeted to a different set of antimicrobial agent substrates. Mutation in regulatory genes can cause increased in expression [9], [10]. This study aimed to evaluate gene expressions of efflux pump (*mexA* and *mexB*) with gene of outer membrane *OprD* under ciprofloxacin stress.

2. Materials and Methods

2.1 Collection samples

Samples collected from different hospitals in Baghdad city, 110 isolates recorded as *Pseudomonas* spp. Morphological and chemical (VITEK system 2 compact) were done to identify *P. aeruginosa.*

2.2 Minimum inhibitory concentration of ciprofloxacin

All bacterial strains subject to double serial dilution method to determine MIC of ciprofloxacin by using Mueller-Hinton 160 microliter broth as the diluent, ciprofloxacin was generated in a micro titer plate starting with a stock concentration of 10 mg/1 ml. The dilutions ranged from 1 to 1024 g/ml. Except for the wells that served as the negative controls, each well was seeded with 10 microliter of a bacterial suspension that was 20 microliter in volume and was comparable to the McFarland standard no.0.5 (1.5 108 CFU/ml). Microtiter plates were kept in an incubator at 37 degrees Celsius for between 18 and 20 hours. Following the incubation period, 20 microliter of resazurin dye was added to each of the wells, and the plates were then returned to the incubator for another 2 hours to check for any color shifts. The Minimum Inhibitory Concentrations were found by visually determining, in broth micro dilutions, the lowest concentrations of the extracts at which the color changed from blue to pink in the resazurin broth assay. This was done to find the Minimum Inhibitory Concentrations (MICs) [11].

2.3 phenotypic detection of Efflux Pump activity under different concentrations of Ciprofloxacin stress and Ciprofloxacin+Ethidium Bromide stress.

Ciprofloxacin and EtBr stain were used for determining whether or not an efflux pump mechanism was present in MHA plates utilizing the EtBr-agar cartwheel (EtBrCW) method. EtBrCW was performed by

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adding EtBr stain in (5,10,15,20,25 µg /ml) to MHA plates containing ciprofloxacin of varying concentrations (32, 64, 125 µg /ml), which were prepared on the same day as the experiment and shielded from light. Following this, the plates were swabbed with A gel documentation device was used to take photographs of the cultures after they were placed on an ultraviolet transilluminator. It was determined what concentration of EtBr was required to produce the least amount of fluorescence in the bacterial mass [12].

2.4 Gene Expression of mexX, oprD and oprM genes

A- Extraction of RNA

By using *TransZol* Up Plus Kit (TRANS/China), RNA was extracted:

B- Removing of Genomic-DNA

By using *EasyScript*® One-Step gDNA Removal and cDNA Synthesis Super-Mix (TRANS/China) kit, genomic DNA was removed.

C- First-Strand cDNA Synthesis

The reaction component (and volume) of this step is summarized as following: Total RNA/mRNA (0.1 ng-5 µg/10 pg-500 ng), Anchored Oligo dT 18 Primer 0.5 µg/µl (1 µl), Random Primer 0.1 µg/µl (1 µl), 2 × ES Reaction Mix (10 µl), EasyScript® RT/RI Enzyme Mix (1 µl), gDNA Remover (1 µl), RNase-free Water (Complete to 20 µl).

D- Assessment of RNA Concentration and Purity

The NanoDrop (Thermo Fisher Scientific, USA) was used to assess the concentration of isolated RNA in order to detect the goodness of samples for a further assessment in RT-qPCR.

F- Gene Expression by RT-PCR

Analysis of gene expression by using RT-PCR was performed for cDNA samples, *mexA*, *mexB* and *oprD* primers that were used in the study [13] with their sequences and normalized with *rpsL* housekeeping gene as list in table 1

primer	sequence	size
mexA	F:5′-ACCTACGAGGCCGACTACCAGA-3′	179 bp
	R:5′-TTGGTCACCAGGGCGCCTTC-3′	
mexB	F:5′-GTGTTCGGCTCGCAGTACTC-3′	240 bp
	R:5′-AACCGTCGGGATTGACCTTG-3′	
oprD	F: 5′-AAGGGGTTCATCGAAGACAGC-3′	108 bp
	R: 5′-GAGCCTTGGGTCCAGTCG-3′	
rspL	F: 5′-CCAACGGTTTCGAGGTTTC-3′	167 bp
	R: 5′-ACCCTGCTTACGGTCTTTGA-3′	

the reaction components was 2×EasyTaq® PCR SuperMix (10 µl), cDNA (2 µl), Primers F+R (2 µl), Nuclease-free Water (6 µl).

Thermal Cycle of RT-PCR of the studied genes was: for *mexB* and *oprD* genes Denaturation 94°C for 10 sec, Annealing 58°C for 15 sec, Extension 72°C for 20 sec., for *mexA* gene Denaturation 94°C for 5 sec., Annealing 68°C for 15 sec., Extension 72°C for 20 sec.

2.5 Statistics Analysis

The statistical analysis was done by using LSD test, one way ANOVA at P value < 0.05 were performed by using GraphPad Prism 7 Statistics software.

3. Results

3.1 Distribution of P. aeruginosa According to Clinical Source

The fifty isolates of *P. aeruginosa* were identified and confirmed, these isolates distributed belong clinical sources as 35 (70 %) burns swab samples, 10 (20 %) from wounds swab, 5 (10 %) from ear discharge. Figure 1 shows Distribution of *P. aeruginosa* isolates according to clinical sources.

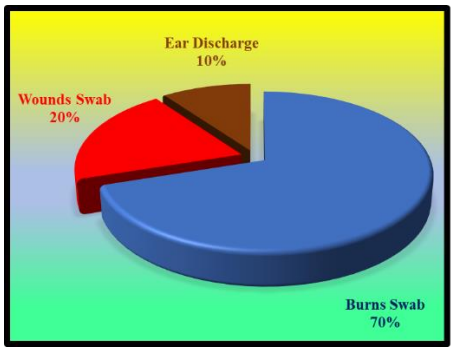

Figure 1: Distribution of P. aeruginosa isolates according to clinical sources

3.2 MIC of ciprofloxacin

The susceptibility of the *P. aeruginosa* isolates against ciprofloxacin in MIC test showed the ranged from 8 to 256 mg/L as it appear in figure 2 According to the established breakpoint values recommended by CLSI (2021), the P. aeruginosa isolates with MIC ≥ 2 mg/L are considered as ciprofloxacin resistant. Nearly, all tested isolates were resistant to ciprofloxacin by MIC test (MIC ≥ 2 and ≥ 16 mg/L respectively).

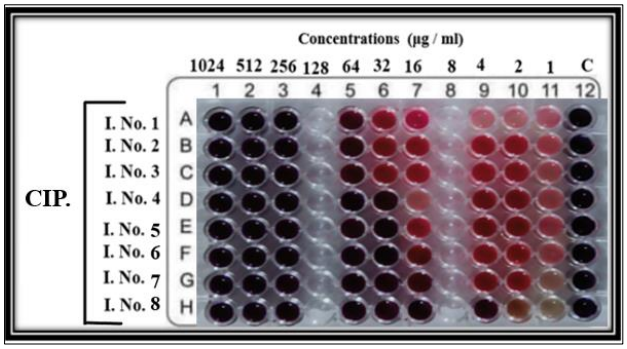

Figure 2: MIC of ciprofloxacin to P. aeruginosa.

3.3 phenotypic detection of Efflux Pump activity under different concentrations of Ciprofloxacin stress and Ciprofloxacin+Ethidium Bromide stress.

The EtBr-agar cartwheel (EtBrCW) method is a practical methodology to assess the presence of efflux activity in large collections of clinical isolates of different bacterial species [4], [12]. This method allows the comparison of different isolates on the basis of their capacity to extrude EtBr. The isolates are streaked in solid media containing increasing concentrations of EtBr and the fluorescence emitted, which is inversely proportional to their capacity to extrude the compound, is compared to the fluorescence of control strains. Using this approach to test a collection of 32, 64, 128 µg/ml ciprofloxacin-resistant *P. aeruginosa*. The current study could discriminate ten isolates in three distinct groups: a group of isolates at the highest 128 µg/ml ciprofloxacin concentration tested with five concentration of Et.Br. 5, 10, 15, 20, 25 µg/ml; a group of isolates at the highest 64 µg/ml ciprofloxacin concentration tested with same concentrations of Et.Br.; and a third a group of isolates at the highest 32 µg/ml ciprofloxacin concentration tested with same concentrations of Et.Br.

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The detection of Efflux pump mechanism was performed using EtBr-agar cartwheel (EtBrCW) method in MHA plates by using Ciprofloxacin (with 3 concentrations 32, 64, 128) and EtBr stain (five concentration with each CIP concentration). The EtBr-agar cartwheel screening method showed efflux activity in 10 MDR strains, table 2 showed the results of EtBr-agar cartwheel methods, figure 3 shows fluorescence of strains because EtBr, which is pumped out by the MexAB- and MexXY-OprM, becomes fluorescent when it enters the cells and binds to nucleic acid [14].

Table 1: Efflux Activity at Varying Concentrations of Ethidium Bromide and Ciprofloxacin (32).

Isolate No.	Conc. of CIP (32)	Control	Conc. of EtBr 5	Conc. of EtBr 10	Conc. of EtBr 15	Conc. of EtBr 15	Conc. of EtBr 20	Conc. of EtBr 25
1	**+ ve**	**Grow**	**- ve**	**- ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**
2	**+ ve**	**Grow**	**- ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**
3	**+ ve**	**Grow**	**- ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**
4	**+ ve**	**Grow**	**- ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**
5	**+ ve**	**Grow**	**- ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**
6	**+ ve**	**Grow**	**- ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**
7	**+ ve**	**Grow**	**- ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**
8	**+ ve**	**Grow**	**- ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**
9	**+ ve**	**Grow**	**- ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**
10	**+ ve**	**Grow**	**- ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**	**+ ve**

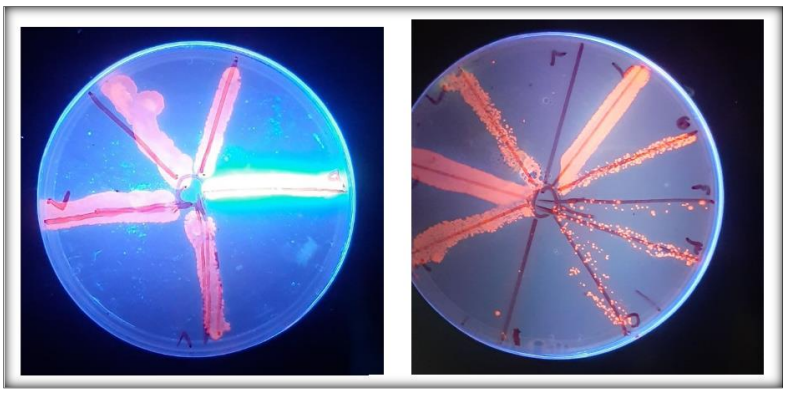

Figure 3: Characterization of clinical isolates according to their efflux capacity.

Fluorescence of *P. aeruginosa* strains on agar plates containing increasing concentrations of EtBr. Cultures were swabbed in MH plates containing increasing concentrations of EtBr. Following overnight incubation at 37˚C for 18 hours, fluorescence was detected under UV light.

3.4 Results of Efflux Pump Expression without stress of ciprofloxacin

A- Results of Housekeeping Gene Expression

Analysis of RNA expression using techniques like real-time PCR has traditionally used reference or housekeeping genes to control for error between samples [15]. The current results show an amplification curve indicating the occurrence of *P. aeruginosa rpsL* housekeeping gene was obtained in samples, *rpsL* Ct results were ranged from 10.03 to 12.13 with average 11.14. Housekeeping genes are usually chosen as internal controls to normalize real-time RT-PCR data [16].

B- Results of *mexA* Gene Expression

Results of *mexA* Ct values were ranged from 32.1 to 36.79 with average 34.33 showed expression compare to *rpsL* (Ct = 11.14) reference gene, as shown in figure 4

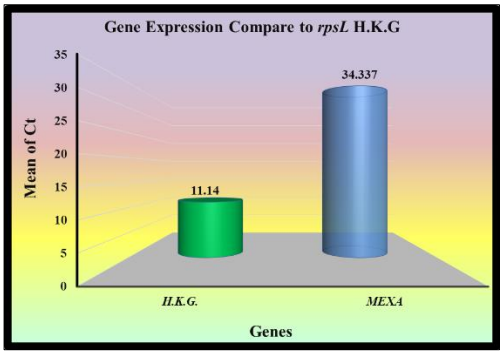

Figure 4: Mean of Ct Values of *mexA* Gene Compare to *rpsL* Reference Gene.

C. Results of *mexB* Gene Expression

Results of *mexB* Ct values were ranged from 14.74 to 19.96 with average 16.791 showed expression compare to *rpsL* (Ct = 11.14) reference gene, as shown in figure 5

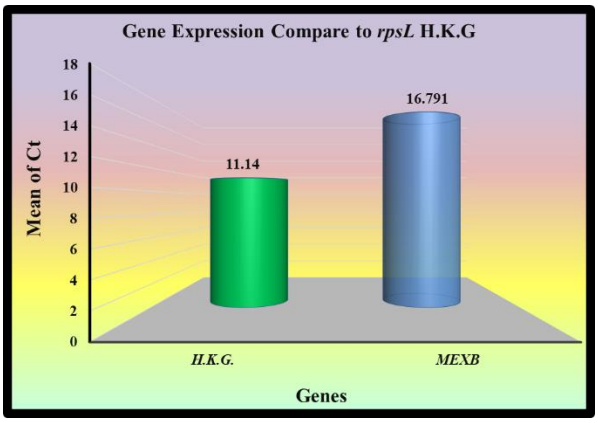

Figure 5: Mean of Ct Values of *mexB* Gene Compare to *rpsL* Reference Gene.

D-Results of *oprD* Gene Expression

Results of *oprD* Ct values were ranged from 20.56 to 24.98 with average 23.141 showed expression compare to *rpsL* (Ct = 11.14) reference gene, as shown in figure 6

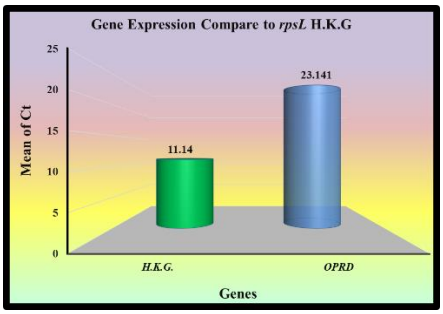

Figure 6: Mean of Ct Values of *oprD* Gene Compare to *rpsL* Reference Gene.

3.4.1 Results of Gene Expression of Efflux Pump in under ciprofloacin only and ciprofloxacin+ Et.Br

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The gene expression of three groups (first was non treated control which was the most effective isolates to CIP, second was treated with CIP 32 µg/mL + Et.Br. 25 µg/mL, and third CIP 64 µg/mL) was used to identify the effect of CIP and Et.Br. on gene expression of *mexA* and *mexB* genes, the results shows that the effective of isolates was reduced compare to non-treated isolates.

The minimum inhibitory concentrations (MICs) of antibiotics are typically at least twice as high for strains of a species that overexpress efflux pumps compared to strains of the same species that do not overexpress efflux pumps. This test is a useful tool for detecting efflux pump overexpression, which is one factor that contributes to multidrug cross-resistance in bacterial populations. Overexpression of efflux pumps was a potential contributing factor in the development of antibiotic resistance in *P. aeruginosa* isolates [20].

A. Results of *mexA* Gene

The results showed that mean of Ct of gene expression was noticed when treated with Et.Br. + CIP and CIP in *mexA* was 37.195 and 34.702, respectively, compare to control 34.337 this may explain that both treatments compare to control not induce the expression of *mexA* genes, on the other hand the gene expression between both treatments was expressed in treatment of CIP 32 compare to CIP+Et.Br

B. Results of *mexB* Gene

The results showed that expression was noticed when treated with Et.Br. + CIP and CIP in *mexB* gene expression was 19.626 and 18.872, respectively compare to control 16.791, this may explain that both treatments compare to control not induce the expression of *mexB* genes, on the other hand the gene expression between both treatments was expressed in treatment of CIP 32 compare to CIP+Et.Br., table 3 shows the Ct values and folding values of treated and control isolates.

C. Results of *oprD* Gene

The results showed that mean of Ct of *oprD* gene expression when treated with Et.Br. + CIP and CIP was 19.23 (with fold of gene expression 15.466) and 17.86 (with fold of gene expression 39.356), respectively, up regulation was noticed compare to control 23.141 (with fold of gene expression 1.000), on the other hand the gene expression between both treatments was approximate in treatment of CIP 32 compare to CIP+Et.Br., Table 3-9 shows the Ct values and folding values of treated and control isolates.

4. Discussion

the development of mechanistic drug resistance in Gram-negative bacteria is greatly influenced by efflux systems. These systems pump dissolved compounds out of the cell, which enables bacteria to alter their internal environment by eliminating potentially harmful substances. These substances include metabolites, antimicrobial agents, and quorum sensing signal molecules. According to the results of the latest research, multidrug-resistant Gram-negative bacteria in Iraq have developed a mechanism of drug resistance that is mediated by efflux pumps [23]. In therapeutically relevant multidrug-resistant Gram-negative bacteria, there is a significant incidence of drug resistance that is mediated by efflux transporters [24], [25]. [26], concluded that *mexA* was detected in all isolates, and Abed, [2], who concluded that *mexA* were detected in 100% (20 isolates) of *P. aeruginosa* isolates. *P. aeruginosa*'s overproduction of mexAB-oprM plays a crucial role in the generation of multidrug-resistant strains since periplasmic membrane fusion proteins (mexA, MFP, mexX, mexC, and mexF) are components of the RND-type efflux pump system, Antimicrobial drug resistance to quinolones, macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin, and beta-lactams has been linked to the MexAB-OprM efflux system [27]. The current study agreed with local study by [26] who concluded that *mexB* was detected in all isolates.

Researchers have established that efflux-mediated resistance exists in a great number of bacterial families. It was discovered before by a number of researchers, increased expression of an efflux mechanism, which is responsible for less antibiotic being accumulated in the organism. *P. aeruginosa*'s Mex efflux pumps are of particular relevance due to the fact that they have an unusually wide substrate specificity. In spite of the fact that the genome of *P. aeruginosa* has been found to contain 12 potential members of this family of efflux systems [4], [28]. Furthermore, the function of an efflux mechanism in fluoroquinolone-resistant clinical isolates has been described for multiple species of bacteria. This active efflux is caused by multidrug resistance (MDR) pumps, which transport foreign-compounds molecules. Some examples of these chemicals include many types of antibiotics and antiseptics, as well as cationic dyes like ethidium bromide (EtBr) and acriflavine [29]. Gene expression of all CIP treated isolates were not over expressed, this may related to the presence of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) as an efflux pump inhibitor, CCCP is a well-known proton motive force inhibitor efflux pump, and it is usually be added to Mueller-Hinton agar while it is being prepared [30], the usage of CCCP as a screening agent in order to determine: first) the prevalence of efflux pump overexpression among multidrug-resistant isolates of *P. aeruginosa*; second) the contribution of efflux pump overexpression as the supposed mechanism for the multidrug cross-resistance between beta-lactams, fluoroquinolones, and aminoglycosides in *P. aeruginosa*; and third) the MIC reduction of beta-lactam, as well as fluoroquinolones, and aminoglycosides in stimulus way with the presence of an efflux pump inhibitor [20]. For ethidium efflux studies, the fluorescence intensity, in addition, CCCP did not impact the EtBr efflux in the strain [4]. This may explain why there was expression in the CIP 32 treatment compared to the CIP+Et.Br. treatment in all of the genes that were investigated (*mexA*, *mexB* and *oprD*).

When a mutant strain of *P. aeruginosa* that lacked its principal multidrug efflux pump complex, MexAB-OprM, was cultured with 100 microM ethidium bromide, the fluorescence that was induced by its binding to DNA upon its entry into cells gradually decreased, as reported by [31]. The scientists reached the conclusion that the internal ethidium bromide "stimulated" either the breakdown of the ethidium bromide or its outflow through the assembly of undiscovered efflux pumps. Because these efflux pumps have a broad substrate specificity and are able to extrude many different antibiotic classes, such as B-lactams, quinolones, and aminoglycosides, it could be a reason to approve the hypothesis that the flouroquinolones (FQ) resistant among *P. aeruginosa* isolated from clinical isolates, particularly from burn wound infections, could be in cooperation with resistance to other existing antipseudomonal agents through overexpression of [32]. It is probable that the widespread usage of FQ agents is one of the primary reasons that *P. aeruginosa* that has been isolated from burn wound infections has developed resistance to FQ drugs. Because of this, extensive use of FQ drugs has a detrimental side effect that contributes to the susceptibility of *P. aeruginosa* to other antipseudomonal medicines through the FQ-selected upregulation of multidrug efflux pumps [33].

Many different bacterial families have been reported to possess efflux-mediated resistance. Increased expression of an efflux mechanism, which is responsible for less antibiotic being accumulated in the organism. *P. aeruginosa's* Mex efflux pumps are of particular relevance because to the unusually broad substrate specificity they exhibit [2], [4].

5. Conclusions

The MIC ciprofloxacin concentration ranged from 8 – 256 µg/ml which can inhibit growth to isolates. The detection of Efflux pump mechanism by using EtBr-agar cartwheel showed that ciprofloxacin concentration 32 µg/ml, EtBr concentration 25 µg/mL were the concentrations that shows fluorescence of *P. aeruginosa* strains in medium, overexpression was notice in expression of genes compare to control.

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