Expression Levels of Efflux pump *mexR* and *norA* Genes in Multi-Drug Resistant in Some Bacteria by Using Quantitative RT-PCR Under Stress of Effect Efflux Pump Inhibitors.

Baidaa Adel Ibrahim[1], Mayadaa Abdullah Shehan[2]

[1]Post Graduate Student, University of Anbar, College of Sciences, Department of Biology, Iraq,
[2]Assist. Prof. Dr. University of Anbar, College of Sciences, Department of Biology, Iraq

Abstract

The study was suggested because of the major role of multidrug-resistant *Pseudomonas aeruginosa and Staphylococcus aureus* in a wide range of clinical infections besides increasing a high resistance against the commonly used difference antibiotic. The efflux pumps have a vital role in multidrug resistance for extruding many toxic materials and antibiotics. This study aimed to measure the expression level of efflux pump genes in MDR *Pseudomonas aeruginosa* and *Staphylococcus aureus* using RT-qPCR technique. In this study, one hundredclinical isolates of MDR*P. aeruginosa* and *S. aureus* isolated from wounds were examined, then 20 isolates selected based on their ability as MDR and wereexposed to different concentrations of Ethidium Bromide (Cartwheel method) to determine the presence of efflux pumps activity. Efflux pump genesmexR, and norA were screened by PCR. The results demonstrated the presence of mexR and norA genesin all MDR isolates. RT-qPCR assay was used for investigating the efflux pump genes expression. The difference in gene expression between active and nonactive efflux pumps was determined when exposed to the antibiotics and efflux pump inhibitors.

Keywords: *Efflux pump inhibitors, mexRgene, norAgene, gene expression, RT-qPCR.*

Introductions

The multidrug resistance is becoming a dangerous problem in the treatment of resistant bacterial infections. The MDR, by definition, is the capacity of pathogens to resist lethal doses of drugs, various in their mode of action and structure, which would be effective in the removal of susceptible isolates of pathogens(1). Among infections caused by Gram-negative rods, *P. aeruginosa* bacteria are the most serious and opportunistic pathogens that cause a high rate of mortality and morbidity in most hospitalized patients(2). Recently, these bacterium infections are caused one of the main problems in hospitals and that are relevant to high rates of mortality, ranging from 18% to 61%(3).

Staphylococcus aureus pathogen remains one of the more serious bacterial pathogens in the public health field because of its high virulence, and also able to cause multiple diseases varying from complicated skin and skin structure infections (cSSSI) to life-threatening situations, like pneumonia, endocarditis and toxic shock syndrome(4).Efflux pumps have revealed as specific key drivers for antimicrobial in both Gram-negative and Gram-positive bacteria, EP also is vital in other different physiological processes like stress-adaptations, pathogenicity, virulence factors, and transportation of necessary nutrients(5). The MDR efflux systems are found in whole bacterial species. These pumps are proteins, which are the capacity of transporting substrate materials with different sizes and properties from the inside cell to the extracellular area of the bacterial cell(6). Efflux pumps are classified into fivefamilies;the resistance nodulation cell division(RND) families, major facilitator subfamily(MFS),small multidrug regulator(SMR), energy-dependent ATP-driven pumps are the ATP-binding cassette (ABC) family and the multidrug and toxic compound extrusion(MATE)(7). *mexR*is a regulatory gene of MexAB-OprM operon and was located upstream of the *mexAB-oprM*operon, also encoding a repressor protein of MarR family, andMexR protein was endowed with oxidation-sensing mechanism which regulates virulence and antibiotic resistance in *P.*

aeruginosa(8). The Efflux pump*norA* is a member of MFS family. Which is a chromosomally encoded protein with 12 transmembrane-spanning segments, also is a proton motive force (PMF) dependent multidrug efflux pump in *Staphylococcus aureus.norA*is a 388 aminoacid protein and reveals to release a diversity of structurally unrelated drugs, like fluoroquinolones, cetrimide, benzalkonium chloride, tetra phenyl phosphonium bromide, ethidium bromide, and acriflavine(7)**.**

Materials and Method

Bacterial Isolates and Identification: Onehundredisolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* isolated from wounds. All bacterial isolates were identified based on morphological characteristics on culture media and biochemical tests (9)**.**Additionally,the diagnosis of all isolates was carried out by the Vitek-2 compact system(Biomerieux, France) using GNR-ID and GPC-ID card(10).

Estimation of Efflux pump activity by Cartwheel method: The cartwheel method was described by (11)to determine the presence or absence of efflux activity within bacterial selected isolates according to the multidrug-resistant pattern. Twenty selected isolates activated on 5ml of (TSB) and incubated at 37°C for 18h. Tryptic soy agar plates containing (EtBr) at concentrations of 0 to 2.5 mg/L, and kept away from light. Bacterial cultures were streaked on EtBr-TSA plates. After chosen a proper concentration, cultures were swabbed on EtBr-TSA plates starting from the center of the plate and spreading towards the edges, Agar plates were incubated overnight at 37 ºC and then examined under U.V transilluminator.

Genomic DNA Extraction: The extraction of DNA was carried out for Twenty *Pseudomonasand Staphylococcus* isolates using DNA Mini kit extraction depending on the instruction of the manufacturing company (Promega, USA).

Detection of the efflux pump genes by Conventional PCR: Uniplex PCR technique was performed to amplify efflux pump genes (mexR and norA) of *Pseudomonas aeruginosa* and *Staphylococcus aureus* respectively. The stock solution of primer was prepared from Lyophilized primers (macrogen,Korea). The specific primers used in this study are listed in Table (1). A typical PCR mixture contained 12.5µl master mix (*Taq* polymerase, MgCl2, PCR buffer and dNTPs), 1µl each of forward and reverse primers, 4µlof DNA and 6.5µl of free water adjusted to a total volume of 25. After centrifuged, the mixture transferred to a thermal cycler to start reaction according to the steps of the suitable program for each gene.

Table (1): Sequences of primers that use for conventional PCR.

Gene		Sequences (5'-3')	Amplicon size (bp)	Reference
norA	F	TGGCCACAATTTTTCGGTAT	182	This study
	R	CACCAATCCCTGGTCCTAAA		
mexR	F	GATTCACGGGGACCTTATCA	162	This study
	R	CGAAGAAGGCAGGCAAAATA		

Total RNA extraction and Synthesis of cDNA: Total RNA was extracted using TRIzoL Reagent RNA Isolation kit (Wizbio, Korea) following the manufacturer recommendations.

By using RT Premix cDNA synthesis protocol (Bioneer, Korea): 15 µl of RNA template and 5 µl Rnase free water were added to theRT PE mix tube. The tube was placed in a thermal cycler programmed. cDNA was performed according to the following procedure; annealing step for 10 min at 37°C, cDNA synthesis for 1 hr at 42°C, heat inactivation for 5 min at 95°C.

Quantitative Real-time PCR (RT-qPCR) assay: Transcription of mexR and norA genes were determined in the 4 MDR *P. aeruginosa* and 4 MDR *S. aureus* isolates. The amplification reaction of the fragment of mRNA was performed with the following master amplification reactionin 10µlof qPCR Mix, 1X, specific primers Table (2); (1µlof forward primers and 1µl of reverse primers), 5µl of cDNA synthesis and 3µl of Nuclease-free Water. Thermocycler Program was used for quantitative RT-PCR as follows: Initial denaturation for 60 sec at 95°C, denaturation for 15 sec at 95°C, annealing/extension for 30 sec at 60°C, the fluorescence

readings were taken after each cycle following the extension step. Then it was followed by a melting curve analysis of 60–95 °C.

A gene *gyrB* housekeeping gene was used as a reference gene. The ΔΔCt method was used for measuring the gene expression. The normalized target amount in the sample was equal to **2-ΔΔCt** and also this value can be used to compare expression levels in the samples(12).

Table(2): Sequences of primers that use to gene expression

Target gene		Primer sequence	Product (bp)	Reference
Gyr Bgene *(mexR)*	F	GGCGTGGGTGTGGAAGTC	187	This study
	R	TGGTGGCGATCTTGAACTTCTT		
Gyr Bgene (*norA*)	F	CCAGGTAAATTAGCCGATTGC	121	This study
	R	AAATCGCCTGCGTTCTAGAG		
norA	F	TGGCCACAATTTTTCGGTAT	182	This study
	R	CACCAATCCCTGGTCCTAAA		
mexR	F	GATTCACGGGGACCTTATCA	162	This study
	R	CGAAGAAGGCAGGCAAAATA		

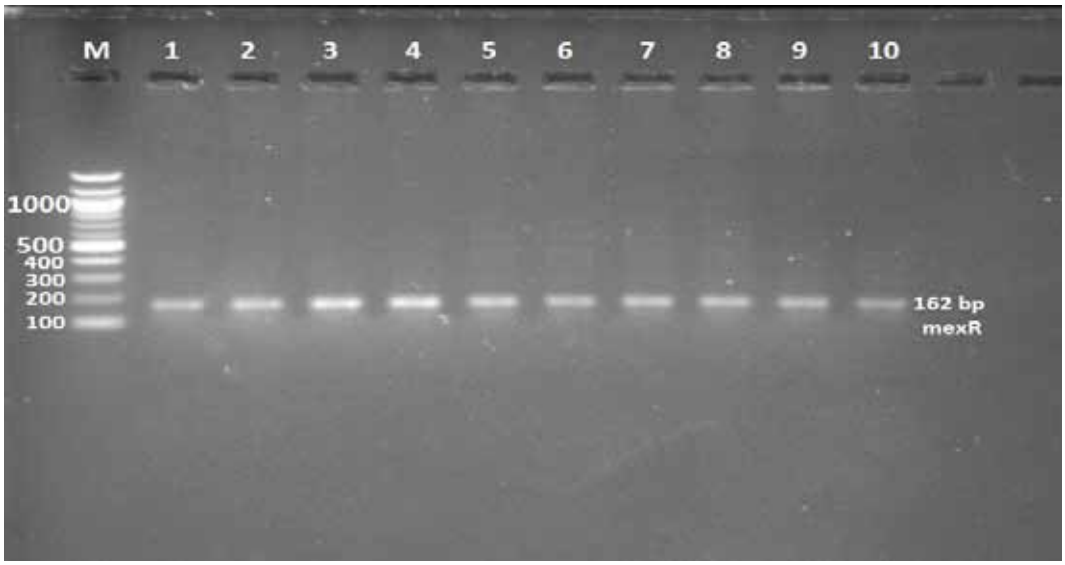

Figure (1): Agarose gel (2%) electrophoresis of PCR products formexR gene (162bp). Lane M: 100bp DNA ladder; lanes 1-10 represent bands of Multidrug resistance *P. aeruginosa* isolates. (75-80V/cm^2 for 2 hr).

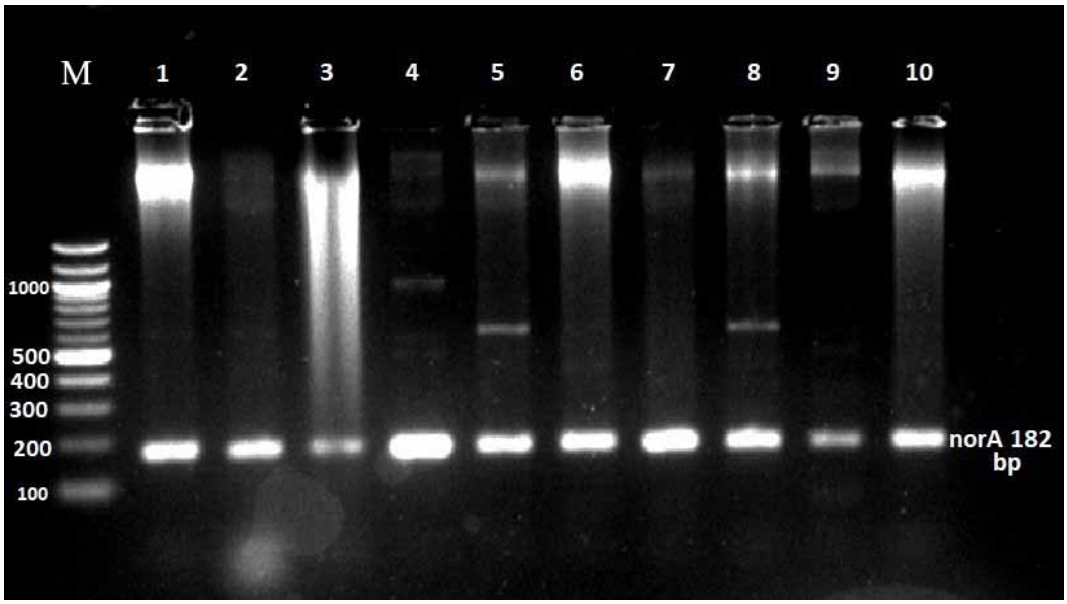

Figure (2): Agarose gel electrophoresis (2%) of PCR products (75-80V/cm^2 for 2 hr) for the efflux pump gene *norA* (182bp). Lane M: 100bp DNA ladder; lanes 1-10: Multidrug resistance *S. aureus* isolates.

Results and Discussion

Efflux pump activity by cartwheel method: The detection of efflux pumps by using different concentration of TSA-EtBr agar plates ranging (0 to 2.5 mg/L), each plate with specific concentration cultured with 20 isolates of *P. aeruginosa and S. aureus* in the form of a cartwheel, after incubation period the result recorded under UV light detects the presence or absence of fluorescence in the mass of bacterial growth, the result identified twenty isolates were grown with higher efflux activity (at concentration 2mg/L). These results were agreed with(13).

Determination of efflux pump genes (*mexR*, *norA)* by PCR: The extracted samples in this experiment have been used to detect the presence of genes encoding efflux pumps and investigation of the genes in bacterial isolates uniplex PCR technique for each DNA. The PCR assay included 20 isolates for the determination of the efflux pump *mexR* and *norA*genes. The PCR products have been confirmed by analysis of the bands on gel electrophoresis. A study by (14)in Egypt,revealed that*mexR* gene was detected in 16 isolates. *mexR* was an important regulatory gene of MexAB-OprM operon,this gene was located upstream of the *mexAB-oprM*operon(15).

Also, the results of the PCR technique for chromosomal efflux pump gene*norA*was observed inisolates of MDR*S. aureus*. In Iraq, a study by(7), out of a total of 96 multidrug-resistant isolates of *S. aureus*, the number of isolates that carried the gene *norA*were 77 isolates (80.21%).

The expression of chromosomal efflux pump genes byRT-qPCR: The major role of this step was to determine the gene expression levels of the chromosomal efflux pump genes in clinical isolates. The presence gene *mexR* and *norA* in bacterial isolates, and compare the gene expression in the presence and the absence of the antibiotics to prove the role of efflux pump genes in the resistance of *P. aeruginosa and S. aureus,* also to compare the gene expression in the presence of the inhibitors to restore the sensitivity of MDR efflux pumps to the antibiotics against which they were developed.

The calculation of gene expression fold change was made using relative quantification(16).For the active efflux pump, the fold of gene expression of *mexR* gene for the three groups (treatments) in addition to untreated group ranged from 1 to 7.86, for an untreated group the fold of *mexR*gene was 1.0. While for the nonactive efflux, the fold of gene expression of mexR gene was ranged from 1to 3.70, also for the untreated group the fold was 1.0. The result of the gentamicin group fold of *mexR* gene (active efflux) was (7.86)and this result was higher (3.70) than the fold of *mexR* gene of (non-active efflux). The PAβN group fold of *mexR* gene (active) was

0.64 and this result was higher (0.47) than the fold of (nonactive). The Phenothiazine group fold of *mexR* gene (active) the result was 1.16 and this result was higher (0.80) than the fold of (nonactive). The results of *mexR* effluxpump gene expression are revealed in table (3).

In this study, when the results of gene expression of fold for antibiotics and synthetic products were compared; it was found that the gene expression was significantly higher when the antibiotics used. Briefly, the gene expression was significantly higher in the gentamicin group than synthetic products, despite the variations in the expression levels of the gene in the gentamicin group, exposure to antibiotics induced the expression of *mexR* gene in the isolates of *P. aeruginosa* studied. Exposure to synthetic products was leading to a decrease in the expression of *mexR* gene in the isolates, except phenothiazine was increased the expression of *mexR* gene in isolates that possess active efflux. The results of gene expression indicated the important role of *mexR* gene in the resistance of gentamicin antibiotics. RND pumps contribute most significantly to antibiotic resistance: MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM, MexBtransports β-lactams; β-lactamaseinhibitorsandcarbapenems (notimipenem), aminoglycosides, fluoroquinolones, tetracyclines, dyes, and several homoserine lactones involved in (QS)(17).

Table (3): Fold of *mexR* expression Depending on $2^{-\Delta Ct}$ Method

Groups	Isolate	Fold of gene expression
Gentamicin	C+D	3.70
	A+B	7.86
PABN	C+D	0.47
	A+B	0.64
Pheno	C+D	0.80
	A+B	1.16
Untreated (Control)	C+D	1
	A+B	1

The mechanism of MexRregulatedantibiotic resistance is due to the formation of intermonomer disulfide bonds in MexR dimer that leads to its dissociation from promoter DNA, also derepression of the mexAB-oprM drug efflux operon, and increased antibiotic resistance of pathogene. PAβN inhibitor was broadly active against MexAB-OprM, MexEF-OprN, MexCD-OprJ, and MexXY-OprMin(18).

Quantitative RT-PCR assay analyzed the mRNA expression of *norA* and compared its expression with isolates without treatment (control), isolates were exposed to azithromycin antibiotic, and also were exposed to synthetic inhibitors. The result of gene expression was shown in table (4). The fold of gene expression of the *norA* gene for active efflux in the isolates of S. aureus studied was (4.61), this result was higher than the fold of nonactive efflux *norA*gene (2.14). The fold of gene expression in isolates exposure to azithromycin was higher than isolates untreated group(control). The fold of gene expression of PAβN was 0.73 for active efflux and for nonactive efflux the fold was 0.40, alsoin isolates that exposures to Phenothiazine fold was 0.87 for active efflux, while for (nonactive) the fold was 0.84 and this results of synthetic products was less than the fold of isolates without treatment group. These results showed that the synthetic products decreased chromosomal efflux pump *norA* gene expression. On the other hand,the above results demonstrate the significant gene expression in isolates exposed to antibiotics.

MDR efflux pump of S. aureus is possessed high resistance to antibiotics, including resistance to b-lactams, aminoglycosides, macrolides, lincosamides, fluoroquinolones, chloramphenicol, sulfonamides,streptomycin, and tetracycline(19). Thioridazine, Phe-Argβ-naphthylamide (PAβN), or the arylpiperazine NMP are some of the compounds categorized were used as efflux pump inhibitors(20).

Table (4) Fold of *NorA* expression Depending on $2^{-\Delta Ct}$ Method

Groups	Isolate	Fold of gene expression
Azithromycin	C+D	2.14
	A+B	4.61
PABN	C+D	0.40
	A+B	0.73
Pheno	C+D	0.84
	A+B	0.87
Untreated (Control)	C+D	1.0
	A+B	1.0

Conclusions

Our study confirmed the important role of efflux pumps in high-level resistance of *Pseudomonas aeruginosa* and *Staphylococcus aureus*isolates, hence the intrinsic resistance to antibiotics of some isolates may

return to their efflux systems. Phe-Arg β-naphthylamide (PAβN) potentiated antibiotic activity through inhibiting *mexR* efflux pump in *P. aeruginosa* and *norA* efflux pump in *S. aureus.*

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

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