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Molecular Detection of *MexA, MexB* Efflux Pump Genes and *MexR* Regulatory Gene in Clinical Isolates of *Pseudomonas aeruginosa*

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Abstract: *Pseudomonas.aeruginosa*, possess the capacity to acquire level of resistance to many drugs. The MexAB-OprM system, is considered one of the most prominent efflux pumps associated with multi-drug resistance. The aims of the study to detect the appearance of *MexA ,MexB* efflux pump genes and *MexR* regulatory gene in *Pseudomonas. aeruginosa* . In this study, we examined seven clinical isolates of *P. aeruginosa* that were isolated from patient's attending (Al-Kindi Teaching Hospital, Shaikh Zayed Hospital, and Imam Ali Hospital).; identified by Vitek 2 compact , Detection *of MexA, MexB* efflux pump genes and *MexR* regulator gene by PCR technique , primers are designed in the college of science / biotechnology department/Iraq. All three of the genes, *MexA, MexB, and MexR*, were existing in all seven clinical isolates of *P. aeruginosa*, according to conventional PCR results. It was concluded that carrying efflux pump genes can make *P. aeruginosa* more resistant to several drugs.

Keywords: MexAB-OprM opern , PCR , *Pseudomonas aeruginosa* , Efflux pump, regulatory gene MexR.

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Introduction

Pseudomonas aeruginosa is an opportunistic pathogen causing severe infections .Its antibiotic resistance, innate and acquired, poses a major challenge in preventing and treating infections caused by this microorganism.(1)**.** Several mechanisms of antibiotic resistance have been associated with *P. aeruginosa*, one of them the production of bacterial efflux pumps are linked to innate resistance, as they move substrates from the bacteria's interior to the cell surface (2, 3) . The five families that make up the efflux pumps are the ATP-binding cassette (ABC) family of energy-dependent ATP-driven pumps, the Resistance Nodulation Cell Division (RND) families, the Major Facilitator Subfamily (MFS), the Small Multidrug Regulator (SMR), and the Multidrug and Toxic Compound Extrusion (MATE) families (4). These crucial RND-type efflux pumps, which are constitutively produced in wild-type bacteria, are in charge of the organisms' inherent resistance to the majority of antibiotics. The example of an efflux pump of the Resistance-Nodulation-cell-Division (RND) type is the MexAB-OprM system (5). The MexAB-OprM pump, an essential excretory system component, can be

inhibited or eliminated. The Subunits of the MexAB-OprM operon in *P. aeruginosa* called MexA, MexB, and OprM were generally supposed to function as the membrane fusion protein, the transporter's body, and the outer membrane channel protein, respectively .the spiral construction of six and seven protomers, which were connected together at one end, revealed unexpected new features in the overall MexA structure. MexA is the membrane bridge protein because The MexA component joined MexB and OprM (6). The MexB subunit is necessary for the pump to function properly; it picks which antibiotics to export, traverses the cytoplasmic membrane 12 times, and is transfer the substrates by utilizing the the energy of proton gradient . An outer membrane lipoprotein known as the OprM component appears to be involved in the last phase of antibiotic release. MexR is the regulater for the expression of MexAB-OprM opern. When the MexR subunit binds to the MexR-MexA intragenic region at the promoter site within the MexAB-oprM operon, it directly inhibits MexAB-oprM expression .These MexA, MexB, OprM, and MexR genes are encoded in the same operon(7). *P. aeruginosa* MexAB-OprM expression is influenced by growth phase , with peaks in late log-phase or early stationary phase. N-butyryl-L-homoserin lactones promote MexAB-OprM expression, inducing virulence factors and indicating quorum sensing regulation (8). When an expression regulator, an assembly of its component parts an energy source, or the outer pores get blocked, the efflux pump is suppressed, which results in antibiotic efflux (9). The purpose of this study is to search if the *MexA ,MexB* efflux pump genes and regulatory genes *MexR* are present in clinically isolated *P. aeruginosa* strains.

Materials and methods

Sample collection

During the period from 4 January 2022 to 28 March 2022 300 clinical specimens including (burn swab, wound swab, ear swab, throat swab, urinary tract infection (UTI) infections, sputum, and urethral swab) were collected from patient’s attending (Al-Kindi Teaching Hospital, Shaikh Zayed Hospital, and Imam Ali Hospital).

Bacterial isolates and identification

The identification of all bacterial isolates was conducted using morphological techniques, which involved the utilization of culture media, including Cetrimide Agar and others media, as well as biochemical assays including oxidase, catalase, TSI, and Indol. **(**10,11**)**.

VITEK2 compact system via identification of *Pseudomonas aeruginosa*

The confirmation of the diagnosis for all clinical isolates was conducted using the Vitek-2 compact system (Biomerieux in France)(12).

Extraction of genomic DNA

The genomic DNA of P.aeruginosa isolates were extracted by commercial PrestoTM Mini gDNA Bacteria Kit (Geneaid, Taiwan).(13) conventional PCR assay was carried out to amplify (MexA,*MexB ,MexR* genes*)* of *P.aeruginosa* by using EasyTaq® PCR SuperMix kit (TransGen,China),as shown in table (1).Primer stock solution was created using lyophilized primers from (Macrogen, Korea),The specific primers for MexA,*MexB and MexR* genes employed in this study are listed in table (2). Following centrifugation, the mixture was sent to a thermal cycler to begin the reaction in accordance with

the instructions of the appropriate program of 30 cycles as shown in (Table 3). Electrophoresis on 1% agarose stained with ethidium bromide was used to analyze the PCR results (Himedia, India), and using a DNA ladder of 100bp as a reference to compare the size of DNA fragments (TransGen,China). Then, Agarose gel was visualized using a UV trans illuminator.

Table (1): Components of PCR reaction with their volume

Components	volume
2×EasyTaq® PCR Super Mix volume (dNTPs, Taq polymerase MgCl2, and PCR buffer)	12.5 µl
Forward Primer (10 pmol/ µl)	1 µl
reverse Primer(10 pmol/ µl)	1 µl
Template DNA	3 µl
Nuclease free water	7.5 µl
Total volume	25 µl

Table (2): Primers sequences to detection efflux pump genes *MexA,MexB and MexR.*

Primer	Primer sequence (5'-3')	Annealing	Product (size bp)	Reference
MexA	**F:**GCAGACGGTGACCCTGAATA **R**:GTATTGGCTACCGTCCTCCA	58	583	*This study Design*
MexB	**F:**GAAGAACTTCCTCATGGTGGTC **R:**GAGGGTCTTCACTACCTCATGG	58	634	
MexR	**F:**AACTACCCCGTGAATCTCGAC **R:**GGCAAACAACTCGTCATGC	58	360	

Table (3): PCR program use to detect (MexA, MexB and MexR) efflux pump genes.

Steps	Temperature	Time	Number of cycle
Initial denaturation	95ºC	5 min	1
Denaturation	95 ºC	45 sec	30
Annealing	58 ºC	45 sec	
Extension	72 ºC	45sec	
Final extension	72 ºC	5 min	1

Results and discussion

Bacterial isolation and identification

From the 300 clinical specimens collected *P. aeuroginosa* recorded highly percentage of isolated (38%), the number and percentage of *P. aeuroginosa* isolates as shown in table(4). All 114 isolates were obtained by cultural characterization and biochemical tests showed in table (5), incubated these clinical isolates on cetrimide agar which is a selective medium for *Pseudomonas spp.*at 37°C for 24 hours and appeared as smooth and greenish to yellow colony with a fruity odor. Finally, only seven clinical *P. aeuroginosa* selected for PCR assay depending on their ability to produce the pyocyanin pigments.

Table (4): The number and percentage of *P. aeuroginosa* isolates from different types of specimens

Type of specimens	No.	Percentage
Chronic burns swabs	40	35.08 %
Wounds swabs	37	32.45 %
Sputum	13	11.40 %
Urine from UTI patients	12	10.52 %
Genital swabs	7	6.14 %
Ear swabs	3	2.63 %
Catheterized patients swabs	2	1.75%

Table (5): Results of Cultural Characterization and Biochemical Tests

Cultural and biochemical tests	Results of *P.aeruginosa*
Gram stain	-
Growth on selective media (cetrimide agar)	+ growth
Lactose fermentation test on MacConkey agar	Non.L.F
Cytochrome oxidase test	+
Catalase test	+
Indole test	-
Motility test	+
Citrate utilization test	+
Urase test	-
Methyl-red	-
Voges- Proskauer	-

(+)= positive result , (-)= negative result, and (L.F)= lactose fermentation

VITEK2 compact system via identification of *Pseudomonas aeruginosa*

In this research we used VITEK2 compact System to comfiem the diagnosis of *Pseudomonas aeruginosa* The probability is approximately 93%-98% that all seven isolated are identified as *Pseudomonas aeruginosa*.

Detection of the *MexA, MexB* efflux pump genes and *MexR* regulatory gene by conventional polymeras chain reaction

The PCR results as figure (1) showed that MexA , MexB efflux pump genes and Mex R regulatory gene exists in all seven isolates *Pseudomonas aeruginosa* which identified by conventional PCR method.our results are different with other studies, such as study in Egypt were MexA, MexB found in 83.3% , 78.6% respectively.(14) other study agreement with our result as this study showed that all there strain of *Pseudomonas aeruginosa* carried out MexA,MexB,and MexR genes (15). In our study findings indicate that The amplified product for each of genes *MexA* , *MexB* and *MexR* were (583bp, 634bp and 360bp) respectively. as shown in figure (1). The detected genes *MexA* ,*MexB* and *Mex R* were validated by electrophoresis on 1% agarose gel at 70 volts for 1.5 hours while stained with ethidium bromide stain and photographed using an ultraviolet (UV) transilluminator.

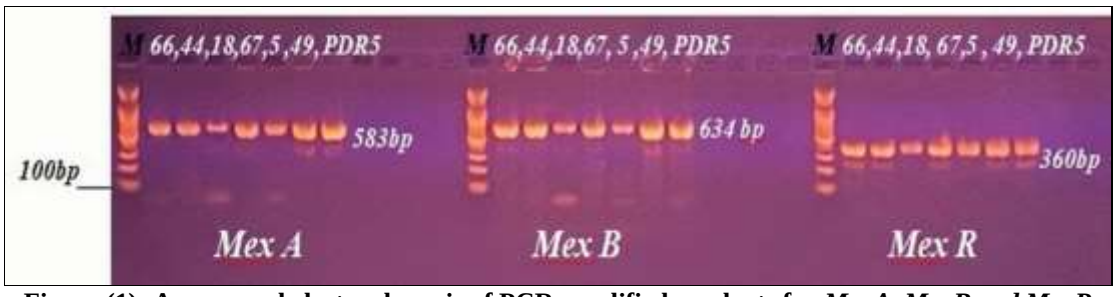

Figure (1): Agarose gel electrophoresis of PCR amplified products for *MexA, MexB and MexR* genes using 1.5 % agarose at 70V/cm for 1.5 houre. lane [M]:marker (DNA ladder 100bp), lanes (66, 44, 18, 67, 5, 49, PDR5)positive results for positive bands of (583bp, 634bp and 360bp) of *MexA, MexB and MexR* genes respectively.

Pseudomonas. aeruginosa is a difficult infection to treat due to the rapid acquisition of drug resistance (16). Efflux pump systems, including RNDs and other drug family pumps, are one of the most antibiotic resistance mechanisms found in *P. aeruginosa*. The MexAB-OprM and MexXY-OprM systems, are examples of efflux pump systems that mediate innate and acquired drug resistance. As the MexAB-OprM efflux pump system is recognized as a naturally occurring drug-resistant mechanism for several antibiotics. It has grown to be the most extensively researched of the efflux pump systems.(17). In this study The results of the polymareas chain reaction assay for MexA ,MexB and MexR genes demonstrated that these genes are present in all clinical isolated of *P. aeruginosa* and that result can led to significant increase in the levels of resistance to the b-lactam antibiotics ceftazidime, cefepime, and piperacillin, specially for these antibiotic because MexAB-OrpM operon is responsible for efflux (expel) of β-lactams and quinolones.(22).The low antibiotic susceptibility is one of *P. aeruginosa* concerning features. This low susceptibility is caused due to the coordinated activity of many drug efflux pumps with many encoded antibiotic resistance genes and the liqimited permeability of bacterial cellular envelopes. In addition to this intrinsic resistance, the altering (mutations) the chromosomally encoded genes for antibiotic resistance determinants, *P. aeruginosa* can quickly acquire resistance to drugs that are physically and functionally different. (18,19) The characteristic of resistance may include intrinsic resistance driven on by various factors, such as limited outer membrane permeability, overexpression of pumps, and development of enzymes that turn medications inactive. The other form of resistance was acquired resistance, which was brought on by mutations or horizontal gene transfer. The third type of resistance is adaptive, which involves the creation of a biofilm layer that serves as a diffusion barrier that reduces antibiotic entry into bacteria. (20, 21).

Conclusion

All *P.aeruginosa* that carry the three genes of efflux pump MexA, MexB and MexR genes in the MexAB-OprM operon in our research, can lead to increase the expel of antibiotic and increase the antibiotic resistance in *P.aeruginosa* ,and that is make a health problem with Patients especially those with impaired immune systems, such as those with severe burns and newborn as well as those with malignancy. On another hand, detection of efflux pump genes can be used to give an idea about a possible emergence of antibiotic resistance. PCR can reduce the identifying process of bacteria with antibiotic resistance to a few hours.

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