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Detection of Efflux pump *MexX* and *MexY* genes in Multidrug Resistant *Pseudomonas aeruginosa*

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Abstract:

High antibiotic resistance among *Pseudomonas aeruginosa* could be viewed as the primary cause of nosocomial infections. In this study, 60 isolates of *Pseudomonas aeruginosa* were isolated from urine, sputum, burn, ear and wounds swab. The major goal was to determine the relationship between *MexX* and *MexY* and the resistance of MDR *P. aeruginosa* to many drugs. Prevalence of multidrug resistant (MDR) *Pseudomonas aeruginosa* was 22 isolates (36.66%) and 38 isolates (63.34%) were found to be non-MDR isolates. Moreover, molecular detection of *MexX* and *MexY* genes was done for MDR isolates.*MexY* gene was found in all MDR isolates and *MexX* gene was detected in 20 (90.9%) MDR isolates. Susceptibility test showed highly significant differences $p \leq 0.01$. Highest resistance was found against piperacillin/sulbactam (45%), followed by ceftazidime/avibactam with 33.33%, and by ceftolozane/tazobactam and levofloxacin which shared same percentage at 31.67%. Almost 23.33% were less resistant to both doripenem and colistin, and 25% to tobramycin. All these findings confirmed a positive correlation between the presence of *MexX* and *MexY* genes and an increase in the resistance for all antibiotics used in this study the results of which were highly significant at $p \leq 0.01$ for all antibiotics. The study found that some antibiotics that had not been studied previously, also had a relationship with the presence of these genes and the resistance of bacteria to antibiotics such as penicillin, unlike it was previously believed that they do not have relation with the efflux pump mechanism in *P. aeruginosa* and that this bacterium use other mechanisms to resist them.

Keywords:*Pseudomonas aeruginosa*, Multidrug resistant, *MexX*, *MexY*, Efflux pump.

الكشف عن جينات مضخة الدفق*MexX*و *MexY* في بكتريا *Pseudomonas aeruginosa*المتعددة المقاومة للمضادات الحيوية

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الخلاصة:

تعتبر المقاومة العالية للمضادات في بكتريا الزائفة الزنجارية السبب الرئيسي لعدوى المستشفيات. في هذه الدراسة تم عزل 60 عينة سريرية من (الادرار، القشع، الحروق، الأذن، ومسحات الجروح). الهدف الرئيسي هو تحديد العلاقة بين جينات *MexX*و *MexY* ومقاومة الزائفة الزنجارية للعديد من الادوية. كانت نسبة الزائفة الزنجارية متعددة المقاومة للأدوية هي 36.66% (22 عزلة). علاوة على ذلك، تم العثور على جين *MexY*

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في جميع العزلات المتعددة المقاومة للأدوية، ووجد جين *MexX* في 20(90.9%) عزلة متعددة المقاومة للأدوية. أظهر اختبار الحساسية فروق ذات دلالات معنوية عند $p\leq0.01$. وكانت أعلى مقاومة ضد piperacillin/sulbactam هي 45%، يليه ceftazidime/avibactam بنسبة 33.33%، يليه ceftolozane/tazobactam وlevofloxacin بنفس النسبة عند 31.67%. وكانت 23.33% هي اقل مقاومة لكل من doripenemو colistin و 25% مقاومة ضد tobramycin. كل هذه النتائج أكدت وجود علاقة ترابط ايجابية بين وجود جينات *MexX*و *MexY* وزيادة المقاومة لجميع المضادات الحيوية المستخدمة في هذه الدراسة و كانت الفروقات المعنويةعالية عند $p\leq0.01$ لكل المضادات الحيوية. وجدت الدراسة بان بعض المصادات الحيوية التي لم تدرس سابقا في الدراسات السابقة مثل البنسلينات التي كان يعتقد بان ليس لها علاقة بجينات الدفق وان بكتريا الزائفة الزنجارية تستخدم اليات اخرى لمقاومتها.

1. Introduction:

P. aeruginosa is a serious pathogen as it has an ability to cause serious infections that can be life-threatening and fatal. With a high rate of morbidity and death, it is one of the most prevalent nosocomial bacteria that harms hospitalized patients. Due to its ability to persist, evolve and develop new mechanisms to resist several antibiotic classes, it has become qualified to cause many serious diseases [1]. One of the major problems being faced by the modern medicine is that it exhibits antibiotic resistance in *P. aeruginosa*, especially MDR, which may be the main factor contributing to the high mortality rates in human [2]. The efflux pump is one resistance mechanism that is intimately linked to the formation of MDR and XDR bacteria as it may transport several drug classes. Four primary sets of efflux pumps have been linked to resistance-ability of *Pseudomonas aeruginosa*: MexXY-OprM (-OprA), MexCD-OprJ, MexEF-OprN, MexAB-OprM [3, 4, 5]. The MexXY efflux system of *P. aeruginosa* is the only multidrug efflux pump operon lacking a coding sequence for an outer membrane factor. It is made up of the PMFP (Periplasmic membrane fusion protein), *MexX* and the RND (Resistance-nodulation-cell division transporter) *MexY*. However, it does collaborate with OprM to generate a multidrug efflux system from the MexAB-OprM operon [6]. The MexXY operon in certain strains, including *P. aeruginosa* PA7 [7], contains the coding sequence for OprA, an additional outer membrane component that can interact with MexXY [8]. Additionally, MexXY has been demonstrated to be crucial for *P. aeruginosa* from cystic fibrosis lung tissue that has a defective MexAB pump and plays important role in resistance against many drugs [9, 10]. MexXY conjunction with OprA is also capable of expelling and conferring resistance to carbenicillin and sulbenicillin [11]. Change in only one amino acid for *MexY* leads to increased resistance not only for aminoglycoside but also for fluoroquinolone and cefepime.These findings confirmed essential role of *MexY* to increase resistance for many drugs for MDR *P. aeruginosa* [10].

2. Materials and Methods

2.1. Isolation and Identification

A total of 156 clinical specimens were obtained aseptically from various sources, including urine, burn swabs, wound swabs, sputum, and ear swabs. The samples were collected from male and female patients of all ages at Al-Yarmouk Teaching Hospital and Al-Mahmoodiya Hospital. Strict sterile conditions were maintained throughout the collection process. Specimens were inoculated by selective and differential media at 37°C and for 24 hrs. Colonies on MacConkey medium that appeared as pale non lactose fermenter colonies were selected and then a single colony was inoculated on cetrimide medium to carry out other tests. Single colonies were selected for diagnosis tests such as biochemical, morphological, oxidase and catalase, and then gram stained [12]. Diagnostic results were confirmed by Vitek 2 compact system.

2.2. Antibiotic Sensitivity Test

Sensitivity card of VITEK 2 compact system (AST-XN20) which included seven antibiotics, was used for this purpose. A volume 145 µl of bacterial suspension was transferred to the antibiotics sensitivity test tubes and then interred to VITEK 2 compact system according to the manufacturing company's protocol [13].

2.3. Molecular Assay:

2.3.1.DNA Extraction and Purification:

DNA extraction and purification was based on ABIOpure™ Total DNA Kit (ABIOpure, USA).

Two pairs of primers were used:

For *MexX* forward primer TGAAGGCGGCCCTGGACATCAGC, reverse primer GATCTGCTCGACGCGGGTCAGCG by Dumas *et al.* [14].

For *MexY* forward primer TCAGGCCGACCTTGAAGTAG, reverse primer TCTCGGTGTTGATCGTGTTC by Serra *et al.* [15].

Reaction setup and thermal cycling protocol, performed using Thermal Cycler, ThermoFisher Scientific, USA, are listed in Tables 1 & 2.

Table 1: Polymerase chain reaction program

Steps	*Temperature °C*	*Time (m: s)*	*Cycle*
Initial Denaturation	**95**	**05:00**	**1**
Denaturation	**95**	**00:30**	**30**
Annealing	**60**	**00:30**	
Extension	**72**	**01:00**	
Final extension	**72**	**07:00**	**1**
Hold	**10**	**10:00**	

Table 2: Polymerase chain reaction components

Master Mix Components	*Volume For 1 Sample*
Master Mix	12.5 µl
Forward Primer	1 µl
Reverse Primer	1 µl
Nuclease Free Water	8.5 µl
DNA	2 µl
Total Volume	25 µl

2.4. Statistical Analysis

All findings were processed statistically using SAS 2018 [16].

3. Results and Discussion

3.1. Isolation and Identification

Accordingly the culture characteristics and other biochemical tests are shown in Table 3, and oxidase test results are shown in Figure 2, and pyocyanin production on cetremide agar is shown in Figure 1. Only 60 isolates were diagnosed as *P. aeruginosa* and then confirmed by Vitek 2 compact system.

Table 3: Culture characteristics and biochemical reactions.

Culture and Biochemical Reaction	*Results*
Growth on Blood Agar	β-haemolysis
Growth on MacConkey Agar	Pale colonies
Growth on Cetramide Agar	Appeared as elevated and blue-green colonies with a grape like odor
Gram Reaction	G-ve and rods
Catalase Reaction	Positive
Oxidase Reaction	Positive
Growth at 42°C	Growth

Figure 1:*P. aeruginosa* on Cetrimide agar

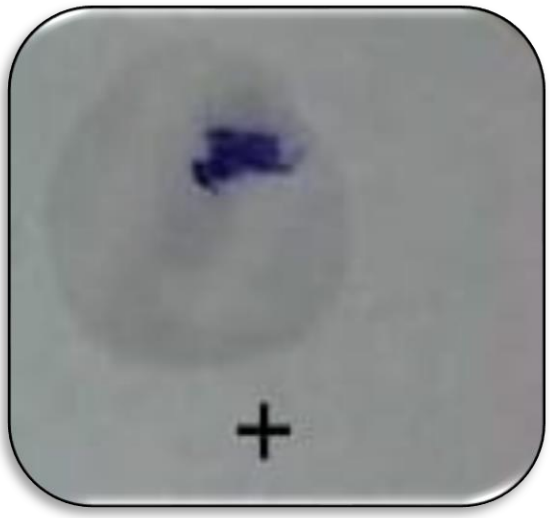

Figure 2: Oxidase test positive for *P. aeruginosa*

The samples were collected from various clinical sources (urine, sputum, burn swabs, wound swabs, and ear swabs), of which 60 (38.46%) specimens were diagnosed as *P. aeruginosa*, based on their findings shown above. This demonstrated a highly significant difference of $p \leq 0.01$, and categorized them into 5 groups(Figure 3). Prevalence of *P. aeruginosa* according to their sources were 40%, 25%, 20%, 10% and 5% specimens from burn, urine, wound, sputum, and ear swab respectively.This result showed a highly significant difference $Pp \leq 0.01$ which agrees with the findings of a recent study conducted by [17].

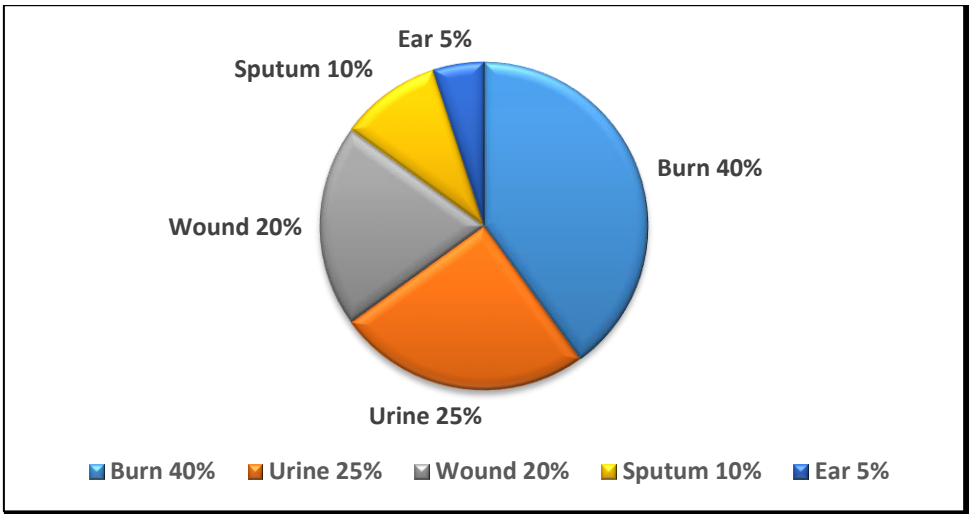

Figure 3: Prevalence of *P. aeruginosa* in different clinical specimens.

Out of 60 isolates, 22 (36.66%) isolates were MDR *P. aeruginosa* which were identified by Vitek-2 technique, at a significant value of $p \leq 0.05$. This result agrees with thefindings of Sulaiman and Abdulhasan in a local study in Baghdad city which showed that only 22 (42%) from all isolates of *P. aeruginosa* were considered as MDR [18].

3.2. Antibiotic Sensitivity

Resistance to 7 antibiotics is at highly significant differences ($p \leq 0.01$). Highest resistance was detected against piperacillin/sulbactam 45%, followed by ceftazidime/avibactam 33.33%, ceftolozane/tazobactam 31.67%, levofloxacin 31.67%, tobramycin 25%, and the lowest resistance levels were 23.33% against both doripenem and colistin (Figure 4).

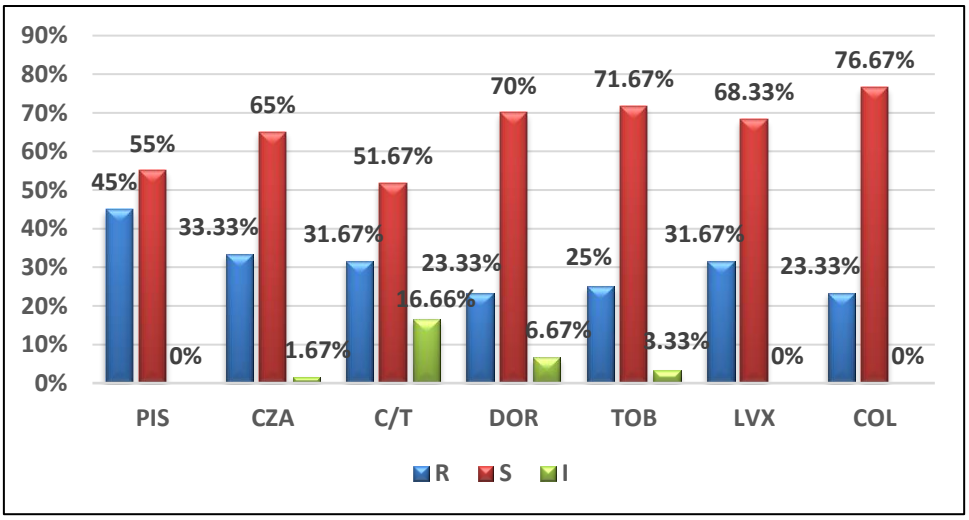

Figure 4: Results of antibiotics sensitivity test. PIS: Piperacillin/Sulbactam, CZA: Ceftazidime/Avibactam, C/T: Ceftolozane/Tazobactam, DOR: Doripenem, TOB: Tobramycin, LVX: Levofloxacin, COL: Colistin, R: Resistance, S: Sensitive, I: Intermediate.

3.3. Molecular Detection of *MexX* and *MexY* Genes:

MexY gene was found in all MDR *P. aeruginosa* isolates. Whereas *MexX* gene was found in in 20 (90.9%) MDR *P. aeruginosa* isolates. Figures 5 and 6show highly significant differences at $p \leq 0.01$.

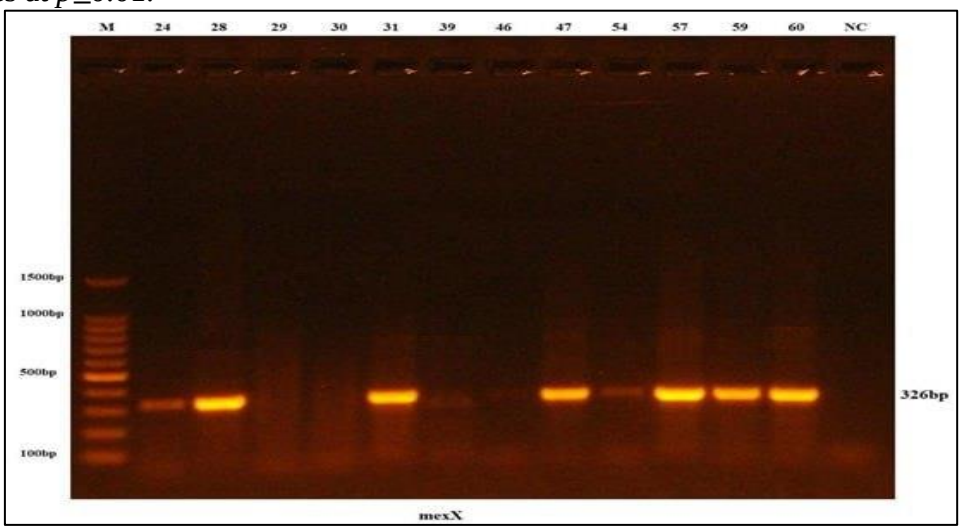

Figure 5: Amplification of *MexX* with 326bp PCR products on 1.5% agarose gel electrophoresis stained by ethidium bromide. M., 100bp ladder marker.

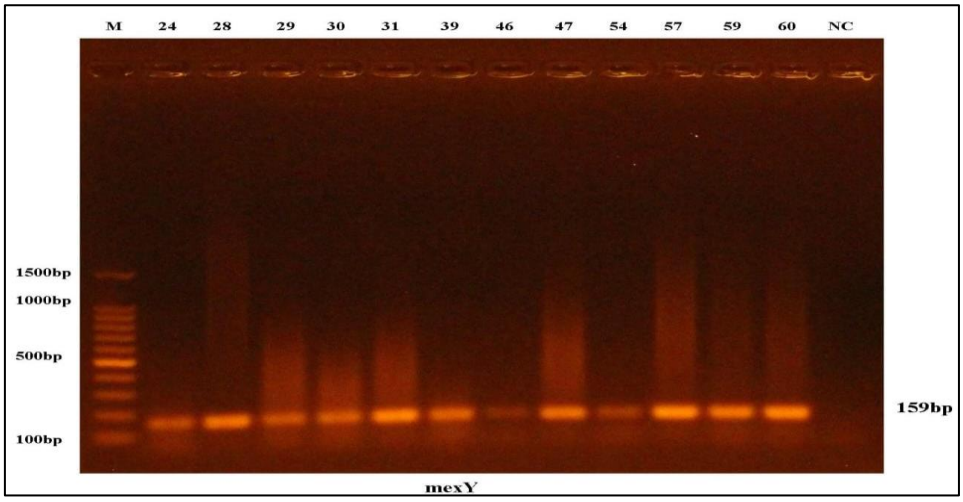

Figure 6: Amplification of *MexY* with 159bp PCR products on 1.5% agarose gel electrophoresis stained by ethidium bromide.M., 100bp ladder marker.

3.4. Relationship of Presence of *MexX* and *MexY*Genes and Resistance to Antibioticsin MDR *P. aeruginosa*:

Recent studies have revealed that the presence of these genes makes bacteria more resistant to antibiotics and the isolates that are sensitive to many antibiotics don't have these genes such as *MexX* gene [19]. The results shown in Tables 4 and 5refer to the relation between antibiotic resistance and the presence of *MexX* and *MexY* genes ($P \leq 0.01$).These results, which were highly significant, confirmed the importance of *MexX* and *MexY* genes in resistance to antibiotics which increases in bacteria with the above mentioned two genes.

Table 4: Role of *MexX* gene's resistance to antibiotics in MDR *P. aeruginosa*

Antibiotic Resistance	*MexX gene*		*Probability*
	Positive (%)	**Negative(%)**	
	Piperacillin/Sulbactam		
R	19 (86.36%)	2 (9.1%)	
I	0	0	0.0001 **
S	1 (4.54%)	0	
	Ceftazidine/Avibactam		
R	18 (81.8%)	2 (9.1%)	
I	0	0	0.0001 **
S	2 (9.1%)	0	
	Ceftolozane/Tazobactam		
R	17 (77.26%)	2 (9.1%)	
I	2 (9.1%)	0	0.0001 **
S	1 (4.54%)	0	
	Doripenem		
R	14 (63.63%)	0	
I	0	0	0.0081 **
S	6 (27.27%)	2 (9.1%)	
	Tobramycin		
R	13 (59.09%)	2 (9.1%)	
I	0	0	0.0086 **
S	7 (31.81%)	0	
	Levofloxacin		
R	14 (63.63%)	2 (9.1%)	
I	0	0	0.0081 **
S	6 (27.27%)	0	
	Colistin		
R	12 (54.54%)	2 (9.1%)	
I	0	0	0.0094 **
S	8 (36.36%)	0	
	***** (p≤0.01).***		

R: Resistance, S: Sensitive, I: Intermediate.

Table 5: Role of *MexY* gene's resistance to antibiotics in MDR *P. aeruginosa*

Antibiotic Resistance	*MexY gene*		*Probability*
	Positive (%)	**Negative (%)**	
	Piperacillin/Sulbactam		
R	21 (95.45%)	0	0.0001 **
I	0	0	
S	1 (4.55%)	0	
	Ceftazidine/Avibactam		
R	20 (90.9%)	0	0.0001 **
I	0	0	
S	2 (9.1%)	0	
	Ceftolozane/Tazobactam		
R	19 (86.36%)	0	0.0001 **
I	2 (9.1%)	0	
S	1 (4.54%)	0	
	Doripenem		
R	14 (63.64%)	0	0.0026 **
I	0	0	
S	8 (36.36%)	0	
	Tobramycin		
R	15 (68.18%)	0	0.0007 **
I	0	0	
S	7 (31.82%)	0	
	Levofloxacin		
R	16 (72.73%)	0	0.0002 **
I	0	0	
S	6 (27.27%)	0	
	Colistin		
R	14 (63.64%)	0	0.0069 **
I	0	0	
S	8 (36.36%)	0	
	***** (p≤0.01).***		

R: Resistance, S: Sensitive, I: Intermediate.

4. Discussion

P. aeruginosa possesses a wide range of innate, adaptive and acquired resistance mechanisms to the antimicrobials used these days. Synergistic usage of these medications results in multidrug resistance which frequently leads to the clinical and hospital failure in providing a successful therapy [18]. Due to the excessive usage of drugs against bacteria,the present study found increased resistance to common drugs. The current findings showed presence of MDR *P. aeruginosa*, which agrees with results of Sweedan's study[19] which found *P. aeruginosa*'s multi resistance to most antibiotics. In this study, isolates showed varied levels of resistance to aminoglycoside class, including tobramycin (25%). This result agrees with the results of Fournier's study that showed 23.8% resistance to tobramycin [20]. Whereas the resistance to piperacillin/sulbactam was 45%.This result disagrees with Wang *et al.*,who showed less percentage of resistance against piperacillin/sulbactam (2.5%) [21]which

was a strong indication of increased appearance of drugs resistance by *P. aeruginosa*. Resistance of islaotes to cephalosporin class including ceftazidime/avibactam was 33.33%. This result agrees with Adámková's research results which showed 34.4% resistance of the isolates to ceftazidime/avibactam [22]. Ceftolozane/tazobactam showed 31.67% resistance to the isolates. This result agrees with the results of O'Neall's study that showed 32.4% resistance against ceftolozane/tazobactam and 33.6% against ceftazidime/avibactam [23]. Carbapenem class, including doripenem, showed 23.33% resistance. This result agrees with the results of Ismail and Mahmuod who showed 18.2% resistance to doripenem antibiotic [24]. Fluoroquinolone class including levofloxacin, showed 31.67% resistance. The results of levofloxacin in this stidy agree with the results of Yang's study that the isolates had 28.5% resistance against levofloxacin [25]. Polymyxin class including colistin showed 23.33% resistance. This result agrees with various studies such as the study conducted by Abd El-Baky that showed 21.3% resistance against colistin [26]. Results of another study showed that *P. aeruginosa* resisted about 70%, 80%, 40% and10% respectively amikacin, ciprofloxacin, ceftazidime and meropenem respectively. However, *P. aeruginosa* was 100% sensitive to imipenem [27]. The results suggest that no longer beneficial uses of these antibiotics as line therapy for infection of *P. aeruginosa*. Importantly, all these findings confirmed a positive correlation between the presence of *MexX* and *MexY* and an increase in the resistance for all antibiotics used in this study, which was highly significant at $p \leq 0.01$ for all antibiotics. These results agree with those of Abd El-Baky *et al.* and Mackenzie *et al.* studies [18, 26] that these genes were present only in resistant isolates and absent in sensitive isolates [18]. *P. aeruginosa* used these genes as effective mechanisms to resist different antibiotics [8, 28]. MexXY conjunction with the *OprA* is also capable of expelling and conferring resistance to carbenicillin and sulbenicillin [9]. Multidrug resistance may result from many factors such as increased efflux pump gene expression, drug enzymatic inactivation, target structural changes and others [29]. It is well known that bacteria use a variety of various resistance strategies. based on the Comprehensive Antibiotic Resistance Database (Card) [30]. Resistant processes include target super-expression, target substitution, creation enzymes which modify drug's molecules, goal modification, deactivating as well as damaging medication, pumps which discharge harmful elements, alterations in pores which inhibit penetration for exterior compounds [31, 32, 33]. As a result of the long-term use of various antimicrobial medicines, bacteria have evolved a number of resistance mechanisms. Multidrug-resistant (MDR) bacteria have emerged as a result of the acquisition of numerous resistance determinants, by selecting mutations that are resistant as well as laterally transferring genes for resistant [34, 35]. Due to the prevalence of the multidrug resistance *Pseudomonas aeruginosa*, and that efflux pump is the important mechanism to resist antibiotics, this study approved that *mexX* and *mexY* multidrug efflux pump in this bacteria had an important role in being resistant to used antibiotics in current study. It was also observed that almost all MDR *P. aeruginosa* isolates that showing resistance to these antibiotic groups carried *MexX* and *MexY* genes.

5.Conclusion:

P. aeruginosa possesses a wide range of innate, adaptive resistance mechanisms to the antibiotics. One of these mechanisms is efflux pump. For that, these bacteria were resistant to almost all antibiotics used in this study. MDR of *P. aeruginosa* harbored efflux pump genes such as *mexX* and *mexY* .This study also found that these genes possessed relationship between them and antibiotics resistance in MDR *P. Aeruginosa* when ($p \leq 0.01$). In other words, if these genes were found in isolates, the bacteria would still be more resistant to antibiotics. And that they had an important role in the resistance of MDR *P. aeruginosa*. Consequently, the increase in the resistance of this dangerous bacterium to antibiotics also increase mortality percentage.

6. Conflict of Interest:

The authors declare that they have no conflicts of interest.

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