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MOLECULAR DETECTION OF THE MEX EFFLUX PUMPS GENES IN EXTENSIVELY DRUG-RESISTANT AND PANDRUG-RESISTANT *PSEUDOMONAS AERUGINOSA* ISOLATED FROM IRAQI PATIENTS IN DIYALA

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Abstract:

The aim of this study was to determine the prevalence of Four clinically-important efflux pumps including MexAB, MexXY, MexCD and MexEF in the Extensively Drug-resistant and Pandrug-resistant *Pseudomonas aeruginosa* isolated from clinical specimens in some hospitals in Diyala using a combination of resistance-phenotypic markers and PCR . This study was carried out during the period from February 2019 to August 2019. Clinical isolates (81) collected from patients suffering different infections. The isolates were diagnosed using classical methods and the VITEK 2 automated system followed by molecular detection using species-specific primer for 16S rRNA. All isolates were tested toward the different class of antimicrobials by using agar diffusion method using 18 clinically important antipseudomonal agents. The results of resistance were as following: piperacillin 74.07, ticarcillin 85.18%, Amoxicillin-Clavulanic acid 93.82%, Ticarcillin/clavulanic acid 71.60%, ceftriaxone 87.65% , cefotaxime 85.18% and ceftazidime 75.30%, cefepime 80.24%, streptomycin 90.12% ,gentamicin 85.18% ,tobramycin 65.43%, amikacin 56.79%, ciprofloxacin 67.90%, levofloxacin 72.83% , oflaxacin 69.13%, Aztreonam 50.61%, Imipenem 11.11% and Meropenem 23.45%. In this investigation, antibiotic susceptibility testing of the *P. aeruginosa* isolates showed that 20(24.69%),25(30.8%), 27(33.33%), and 9(11.11%) of the isolates were MDS,MDR, XDR and PDR, respectively. Based on the results from PCR, out of ninteen *P.aeruginosa* isolates(XDR and PDR), 19(100%) gave positive results for efflux system *MexY* gene , 18 isolates (94.7 %) have *MexB* and *MexF* genes, while 17 isolates (89.47%) have *MexD* gene. This may indicate the prevalence these type of resistance in the current isolated bacteria.

Keywords: P. aeruginosa, XDR, PDR, Efflux Pumps.

INTRODUCTION

Pseudomonas aeruginosa strains, especialy multidrug-resistant, have caused serious problems in many countries, including Iraq.The increasing prevalence of nosocomial infections produced by multidrug-resistant (MDR) ,extensively drug resistant (XDR) and pandrug-resistant (PDR) *Pseudomonas aeruginosa* strains poses a grim challenge for antimicrobial therapy (El Zowalaty *et al*., 2015). *P. aeruginosa* is an opportunistic pathogen involved in many infections worldwide, such as respiratory infections, urinary tract infections, hospital-acquired pneumonia, wound and soft tissue infections, and bacteremia in immunocompromised patients, including patients with thermal injuries (Al-Azawy *et al*.,2013; Weiner *et al*.,2016). *P. aeruginosa* is divided into different phenotypes based on the drug resistance patterns of the organism(Gill *et al*.,2016).Multidrug-resistant (MDR) phenotype is defined as *P. aeruginosa*, which is resistant to more than one antimicrobial agent in three or more antimicrobial categories. A similar resistance to more than one antimicrobial agent in <3 antimicrobial categories is defined as drug-resistant (DR) *P. aeruginosa*. Extensively drug resistant (XDR) phenotype is defined as *P. aeruginosa*, which is resistant to more than one antimicrobial agent in all the antimicrobial categories, except in two or less. Pan- drug resistant (PDR) phenotype is defined as a bacterium which is resistant to all antimicrobial agents in all antimicrobial categories(Magiorakos,2011).

Pseudomonas aeruginosa infections are problematic due to its intrinsic as well as acquired resistance to many effective groups of antibiotics. Intrinsic MDR *P. aeruginosa* is attributed by limited permeability of

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outer membrane, production of inducible β- lactamase and Multidrug Efflux system. (Mohamad *et al.*,2017). Among four MDR efflux system in *P. aeruginosa,* MexAB-OprM and MexXY-*Opr*M contribute to intrinsic resistance whereas hyperexpression of MexCD-*Opr*J and MexEF-*Opr*N leads to acquired MDR *P. aeruginosa* (Hassuna *et al.*, 2015).

The prevalence of multidrug-resistant *P. aeruginosa* (MDRP) non-susceptible to quinolones and aminoglycosides in addition to beta-lactams is reported worldwide (Saderi *et al.*,2015). Carbapenems are β -lactam antibiotics. It binds to pencillin binding proteins (PBP) and hinder the production of cell wall of microorganism (El-Gamal *et al.*,2017).It acts as inhibitors of the enzymes of PBP. At the end cell death of microorganism occurs due to osmotic pressure.(Chow *et al.*,2016).

However, little information is available on the distribution of *MexB,MexY,MexD* and *MexF* producing isolates and colonal infections with these isolates in Diyala/Iraq, The aim of this study was to determine the prevalence of Mex Efflux-genes (*MexB*, MexY, *MexD* and *MexF*) encoding Efflux pumps among XDR and PDR *P. aeruginosa* isolated from clinical specimens in some hospitals in Diyala.

MATERIALS AND METHODS

Isolation and Identification of Bacterial Isolates

A total of (326) clinical specimen from both gender with different age were collected from the beginning of February 2019 to the end of August 2019, from patients in different hospitals of Baquba city. The isolates were identified by their colony characteristic, gram-stain and confirmed by the pattern of biochemical profiles using Vitek 2-GN system.

Antibiotic Susceptibility Testing

To estimate potential resistance of *P. aeruginosa* isolates against 18 items of antibiotics from different classes, all isolates had been subjected to antibiogram test according to Clinical and Laboratory Standards Institute guidelines (CLSI-2017), and this assay could be preferable achieved by widespread Kirby-Bauer disk diffusion technique was carried out by using disks (Bioanalyse/Turkey) on Mueller Hinton agar: Pipracillin (PRL), Ticarcillin (TIC), Amoxicillin-Clavulanic acid (AMC), Ticarcillin/clavulanic acid (TCC), Cefotaxime (CTX), Ceftriaxone (CRO), Ceftazidime (CAZ), Cefepime(FEP), Ciproflxacin (CIP), Levofloxacin (LEV),Oflaxacin (OFX), Gentamicin (CN), Amikacin (AK), Tobramycin (TOB), Streptomycin (S), Aztreonam (ATM) , Imipenem (IMP) and Meropenem(MEM). Detection of *P. aeruginosa* phenotypes based on the drug resistance patterns. Multidrug-resistant (MDR) phenotype is defined as *P. aeruginosa*, which is resistant to more than one antimicrobial agent in three or more antimicrobial categories. While, the definition of extensive drug resistance (XDR) is an isolate that is resistant to all but one or two classes. PDR when isolates non-susceptible to all seven antimicrobial categories tested.

DNA Extraction and polymerase chain reaction (PCR) amplification

Genomic DNA was extracted from isolates using extraction Kits of Genomic DNA, Purification depending on instruction of manufacturing company (Promga USA). All XDR and MDR isolates were screened by standard PCR conventional using specific primers for *16S rRNA ,MexB, MexY, MexD and MexF* genes as shown in (Table 1).

Table (1):The primers used for Efflux pumps genes detection.

Primer	Oligo sequence (5'-3')	Product size bp	Reference
16S rRNA	F-5` GGG GGA TCT TCG GACCTCA3` R-5` TCC TTA GAG TGCCCA CCCG3`	956	Spilker *et al.* (2004)
MexB	F-5`-ACTTCTTCAGCTTCAAGGAC-3` R-5`-GAGCATGAGGAACTTGTTG-3`	155	Poonsuk and Chuanchuen, 2014
MexD	F-5`-CTACCCTGGTGAAACAGC-3` R-5`-AGCAGGTACATCACCATCA-3`	250	Poonsuk and Chuanchuen, 2014
MexF	F-5`-CATCGAGATCTCCAACCT-3` R-5`-GTTCTCCACCACCACGAT-3`	350	Poonsuk and Chuanchuen, 2014
MexY	F-5`-TCGCCCTATTCCTGCTG-3` R-5`-AGTTCGCTGGTGATGCC-3`	117	Fazeli *etal.*,2014

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The PCR conditions started with thermocycler program according to showed in the (Table 2). Amplified PCR products were detected by agarose gel electrophoresis.

Table (2): Programs of PCR thermocycling conditions for Primers .

Genes Monoplex	Initial denaturation	Denaturation	Annealing	No of cycle
16S rRNA	95ºC / 5 min	95ºC/30 sec	54°C/30sec	30
MexB	95ºC / 5 min	95ºC/30 sec	54°C/30sec	30
MexD	95ºC / 5 min	95ºC/30 sec	54°C/30sec	30
MexF	95ºC / 5 min	95ºC/30 sec	54°C/30sec	30
MexY	95ºC / 5 min	95ºC/30 sec	60°C/30sec	30

* Elongation in 72ºC\ 1 min and final extension 72°C\ 7 min for all genes.

Statistical Analysis

Statistical analysis was performed with Graph Pad Prism version 6 software, percentages were used for the comparison between samples of the study. Data analysis was done using Chi-square for the comparison of categorical data.

RESULTS AND DISCUSSION

A total of 81 clinical isolates of gram negative bacteria primary identified as *Pseudomonas aeruginosa* were collected from different clinical sources.The source of these isolates were as follows in (Table 3) .

Table (3): No.of *P. aeruginosa* isolates according to source of samples.

Type of infection (specimens)	No. of samples &(%)	No. of *P. aeruginosa* &(%)	Percentage of isolates to samples
Wound Infection	98 (30.06%)	24(29.62%)	24/98(24.48%)
Burn Infection	76 (23.31%)	21 (25.92%)	21/76(27.63%)
Ear Infection (Swab)	50 (15.33%)	13 (16.04%)	13/50(26%)
Urine	69 (21.16%)	17 (20.98%)	17/69(24.63%)
(Sputum)	33 (10.12%)	6 (7.40%)	6/33(18.18%)
Total	326 (100%)	81 (100%)	81/326(27.6%)

All isolates were identified through morphological , cultural and some biochemical tests and using Vitek-2-GN system followed by molecular detection using species-specific primer for 16S rRNA(Figure1).

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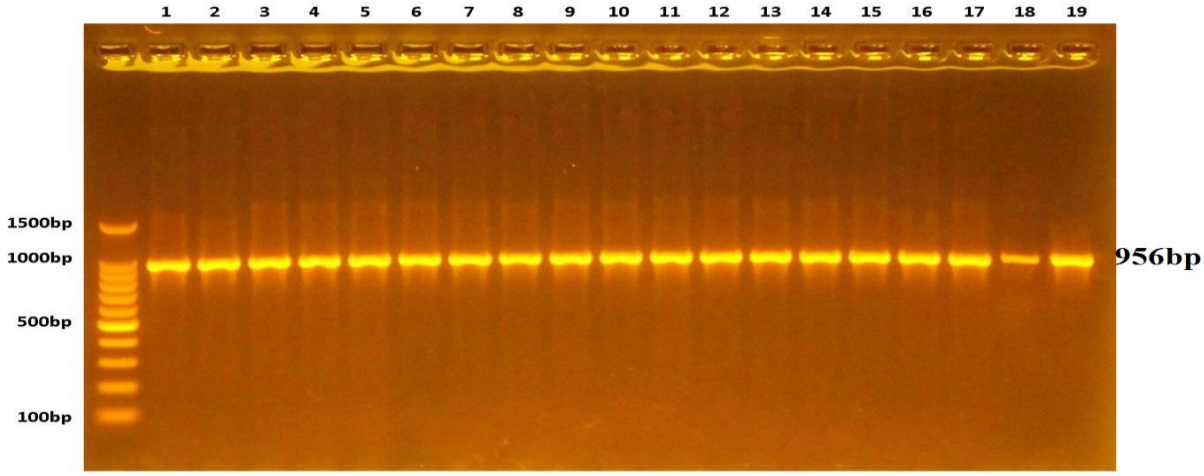

Figure(1): Gel electrophoresis of amplified PCR product for the detection of *16SrRNA* gene (956bp) run on 1%agarose (90 min at 70 volt), stained with ethidium bromide, lane 1-19 *P.aeruginosa* isolates; M:Marker DNA ladder(100bp).

Antimicrobial sensitivity test

Eighty one *P. aeruginosa* isolates were screened for their resistance to 18 different types of antibacterial agents . Results in (Table 4) show that isolate varied in their resistance and sensitivity to the antibiotics.

Table (4): Antibiogram susceptibility of *Pseudomonas aeruginosa* isolates

Antibiotic		Resistant isolates No. & %	Intermediate isolates No. & %	Sensitive isolates No. & %	*p-value*[a]
Class	Type				
1	Pipracilin	60 (74.07%)	5 (6.17%)	16 (19. 75%)	0.008
	Ticarcillin	69 (85.18%)	3 (3. 70%)	9 (11.11%)	0.005
2	Ticarcillin/clavulanic acid	58 (71. 60%)	6 (7.40%)	17 (20.98%)	0.009
	Amoxicillin/Clavulanic acid	76 (93.82%)	1 (1. 23%)	4 (4.93%)	0.001
3	Cefotaxime	69 (85.18%)	4 (4.93%)	8 (9.87%)	0.005
	Ceftriaxone	71 (87.65%)	5 (6.17%)	5 (6.17%)	0.004
	Ceftazidime	61 (75.30%)	2 (2.46%)	18 (22.22%)	0.008
	Cefepime	65 (80.24%)	4 (4.93%)	12 (14.81%)	0.017
4	Ciprofloxacin	55 (67.90%)	-	26 (32.09%)	0.073
	Levofloxacin	59 (72.83%)	-	22 (27. 16%)	0.056
	Oflaxacin	56 (69.13%)	7 (8.64%)	18 (22.22%)	0.042
5	Gentamicin	69 (85.18%)	2 (2.46%)	10 (12.34%)	0.008
	Amikacin	46 (56.79%)	8 (9.87%)	27 (33.33%)	0.051
	Tobramycin	53 (65.43%)	11 (13.58%)	17 (20.98%)	0.038
	Streptomycin	73 (90.12%)	2 (2.46%)	6 (7.40%)	0.007
6	Aztreonam	41 (50.61%)	10 (12.34%)	30 (37.03%)	0.064
7	Imipenem	9(11.11%)	7(8.64%)	65(80.24%)	0.023
	Meropenem	19 (23.45%)	12 (14.81%)	50(61.72%)	0.038

a: *P-value* was calculated using the Chi-square test in terms of the R, I & S group.

†class(1)::[Penicillins-Ureidopenicillin, Penicillins-Carboxypenicillin].(2): β-Lactam-β-lactamase inhibitor combinations Sub-class(3) : [Cephalosporines G^{III}, G^{IV}] class (4): [Fluoroquinolones]. class (5): [Aminoglycosides]. Sub- Sub-class(6):[Monobactams] Sub-class(7): [Carbapenems]. (CLSI, 2017). Classes=class and sub-class.

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It was found high resistance to beta-lactams , aminoglycosides and flouroquinolones. Resistance to monobactam was moderate when 50.61% of isolates being resistant to aztreonam. While the lowest resistance was observed for carbapenems. Profile of antibiotic resistance to other antibiotics is shown in table (4). *Pseudomonas aeruginosa* isolates across countries are increasingly resistant to a higher number of antimicrobial agents. Ali (2016) described that among 60 isolates of *P. aeruginosa*, 30% resist to 14 different antibacterial agents. Astudy by Tawfeeq *et al.* (2017) in Iraq revealed that resistance percentage to Cefotaxim (60.34%) and Pipracillin (59.48%). Results conducted in current study agreement with Al-Wasity ,2018 who reported that *P.aeruginosa* isolates from Baghdad hospitals developed resistance to different antibiotic classes, including flouroquinolones in high resistance rates were 64.5% , 74.2% of isolates resistant to ciprofloxacin and Levofloxacin, respectively.

Based on the results from susceptibility testing, 19 (23.45%) of *P. aeruginosa* isolates were found to be resistant to at least one of carbapenems. Imipenem showed better activity (72.83%) than meropenem (61.72%) in study period. Resistance for carbapenems by disk diffusion was originating in 9(11.11%) isolates for both meropenem and imipenem, and in 10 (12.34%) isolates for meropenem alone, respectively.

Multi-drug Resistance of *Pseudomonas aeruginosa* Isolates

The incidence of Multi-drug resistance (MDR), extensive drug resistance (XDR) and pan-drug resistant (PDR) was investigated among isolates of 81 *P. aeruginosa*. By present definition of MDR, 25(30.8%) of isolates were confirmed as MDR(Table 5), 6/25 (24%) of the them were resistant to three classes of antibiotics, and the remaining (76%) MDR isolates were resistant to four tested classes of antimicrobials. The most common agents involved in MDR were β-lactam/ β-lactamase inhibitor combination, cefotaxime, and gentamicin .However, no MDR isolates was resistant to imipenem.

Among these isolates, 27(33.33%) were resistant to 5 or 6 classes of antibiotics meeting criteria for XDR organisms. The majority of XDR isolates (17 of 27) were resistant to 5 classes of antibiotics, accounting for 63% of all isolates, (and 10 of 27) XDR isolates (37%) were resistant to 6 classes of antibiotics agents, while this 10(37%) of the isolates showed a pattern of XDR including meropenem, β-lactam/ β-lactamase inhibitor combination agents, cephalosporins and monobactams, isolates were susceptible only to imipenem.

Table (5):Multidrug resistance (MDR), Multidrug sensitive (MDS), extensive drug resistance (XDR) and pandrug resistance (PDR) of *Pseudomonas aeruginosa* isolates(n=81).

Type of resistance	No. of *P. aeruginosa* &(%)		No. of resisted Antibiotics classes(n=7)
MDR	25(30.8%)	6/25	3
		19/25	4
XDR	27(33.33%)	17/27	5
		10/27	6
PDR	9(11.11%)		7
MDS	20(24.69%)		

Moreover, 9(11.11%)of isolates were resistant to all antibiotic classes (seven), and hence considered as PDR isolates. Figure(2) shows the isolate as PDR *P. aeruginosa*.

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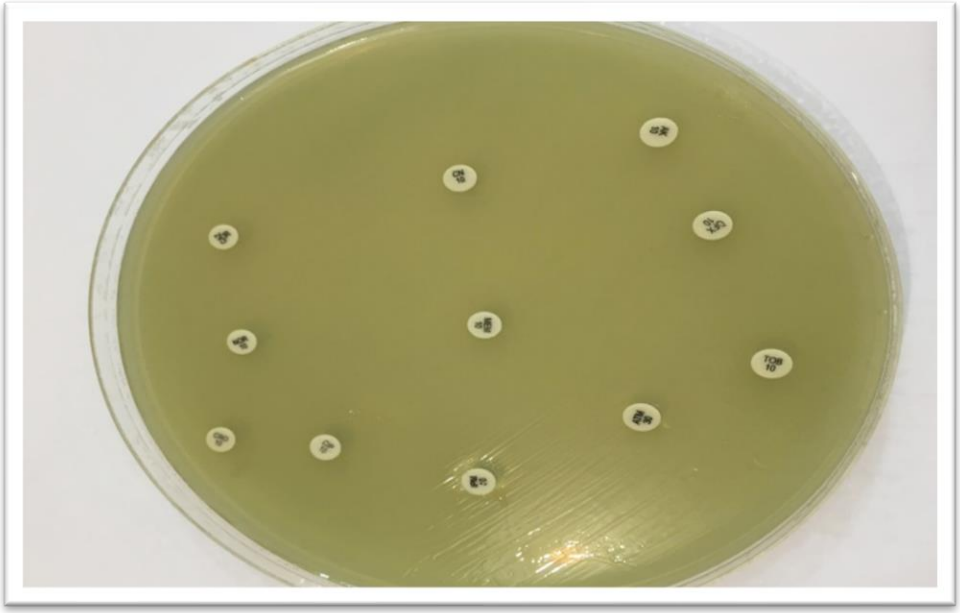

Figure (2): Disk diffusion test for *P.aeruginosa* isolate apan-drug resistant (PDR).

In this investigation, antibiotic susceptibility testing of the *P. aeruginosa* isolates showed that 20(24.69%),25(30.8%), 27(33.33%), and 9(11.11%) of the isolates were MDS,MDR, XDR and PDR, respectively. Al-Wasity , (2018) reported that 14.5%% of *P. aeruginosa* isolates from clinical samples obtained from hospitals in Baghdad/Iraq were pan drug resistant (PDR) and 29% of isolates were Multidrug sensitive (MDS) which is in a good agreement with our results that among 81 isolates 11.11% and 24.69% were pan drug resistant (PDR) and Multidrug sensitive (MDS) respectively .

This study reported significantly higher rate of extensive drug resistance -XDR *P. aeruginosa* in Diyala hospitals. By current definition of XDR, 33.33% of isolates were documented as XDR. Bacteria that are classified as XDR are epidemiologically significant due not only to their resistance to multiple antimicrobial agents, but also to their ominous likelihood of being resistant to all, or almost all, approved antimicrobial agents. (Palavutitotai *et al.,* 2018).There are currently very few reports on the clinical outcome of patients suffering from infection caused by PDR *P. aeruginosa*. These suggest that the mortality is high. In conclusion, the high incidence of MDR, XDR and PDR observed among *P. aeruginosa* isolates underlines the strict consideration in antibiotics use at Diyala hospitals. Therefore, it is important to perform antibiotic surveillance programs for appropriate empirical therapy and infection control practices.

Molecular Detection of Efflux Pumps Genes *MexY, MexB, MexD* and *MexF*.

In current study, the result of efflux pumps genes(*MexY*, *MexB*, *MexD* and *MexF*) by two conventional PCR techniques: uniplex and multiplex PCR, for 19 selected isolates showed that all isolates 19 /19 (100%) were positive for one or more efflux pumps.

Uniplex results by PCR technique, showed 19/19 (100%) of isolates were positive for *MexY* in (Figure 3). Based on the results from Multiplex PCR, 18 isolates (94.7 %) have *MexB* and *MexF* genes, while 17 isolates (89.47%) have *MexD* gene figure (Figure 4).This may indicate the prevalence these type of resistance in the current isolated bacteria . *MexY* gene was detected in 8/28 (28.5%) isolates in a study performed by Al-Jubori *et al.* (2015) and this result was disagreed with current study.

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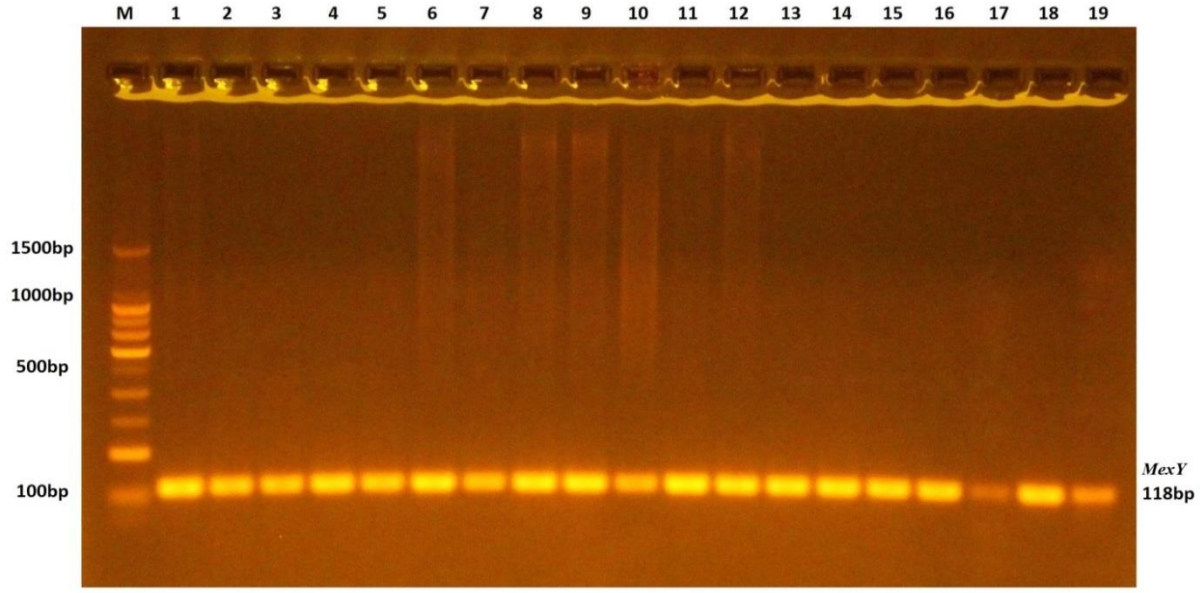
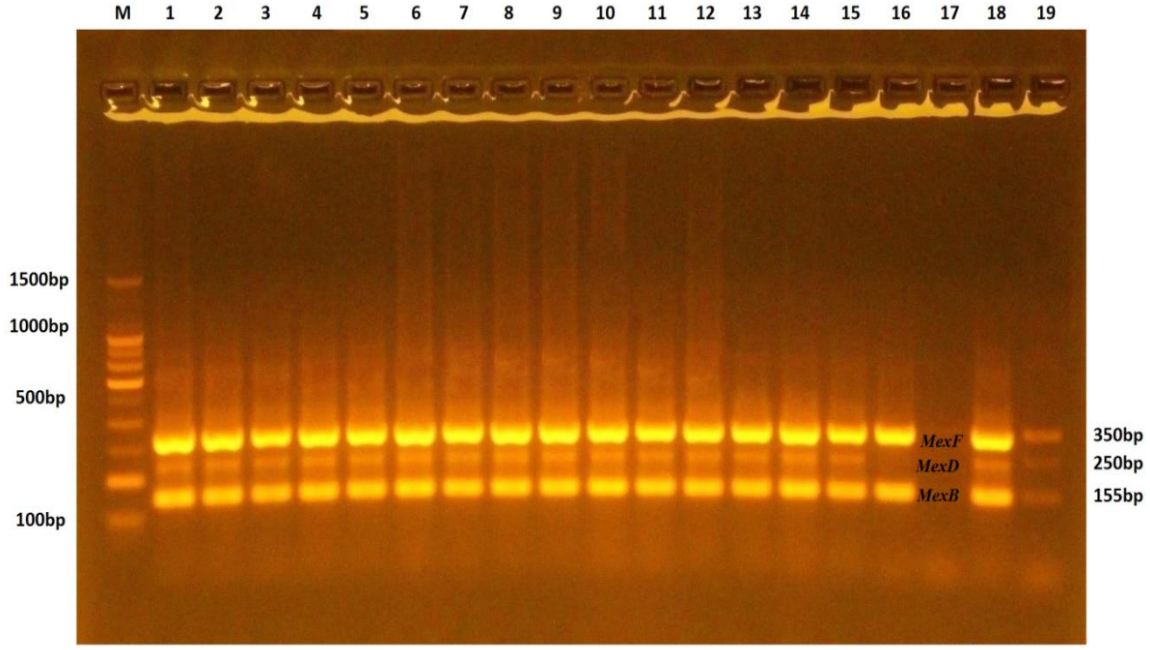


Figure (3): Agarose Gel electrophoresis of amplified PCR product for the detection of MexY gene (118bp) run on 1%agarose (90 min at 70 volt) , stained with ethidium bromide, lane 1-19 *P. aeruginosa* isolates;M: Marker DNA ladder (100bp); All Lanes positive for MexY gene.



Figure(4): Agarose Gel electrophoresis of amplified multiplex PCR product for the detection of MexF(350bp), MexD(250bp) and MexB(155bp) genes run on 1%agarose (90 min at 70 volt) , stained with ethidium bromide, lane 1-19 *P. aeruginosa* isolates;M: Marker DNA ladder (100bp).

Al-Marjani *et al*. (2015) investigated survey of efflux pump gene expression of *P. aeruginosa* in 27 colistin resistant isolates isolated from Iraqi patients. Their investigation included PCR assay for determining the known genes expression of efflux pump as well as *mexY* gene, which illustrated that the prevalence rate of *mexY* was 88.9% and this result was approximated in line of the current study.

The current results showed that 94.7 % percentage of resistant *P. aeruginosa* isolates have *MexB* gene, the

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percentage of *MexB* gene in the current study was higher than previous study in Iran was noted *MexB* appeared in 53.3 % of isolates (Pourakbari *et al*., 2016). In another study in Some Hospitals in Bagdad Governorate (100%) of isolates carried *MexB* gene, this result agreed with current study (Abd ,2018).

At present, the efflux pump has been recognized as one of the significant complexes involved in resistance to most of the classes of antibiotics (Shigemura *et al*.,2015). There are rare reports on prevalence of efflux pump genes in our Provence. In the present study the increased expression level of *MexD-OprJ* genes of efflux pump simultaneously was 89.47% which was relatively more than Murugan *et al*.(2017) findings in India was noted *MexD* gene appeared in 43% of isolates .

In this study, 28 of 45 patients (62%) showed an increased expression level of efflux pumps *MexF-OprN* genes that was higher than previous study in Spain reported *MexF* appeared in 4.2% of isolates (Cabot *et al*., 2011). The multidrug efflux system MexEF-OprN is produced at low levels in wild-type strains of *Pseudomonas aeruginosa*. However, in so-called *nfxC* mutants, mutational alteration of the gene *mexS* results in constitutive overexpression of the pump, along with increased resistance of the bacterium to chloramphenicol, fluoroquinolones, and trimethoprim(Juarez *et al*.,2017).

CONCLUSIONS

In conclusion ,our study showed the high prevalence of Mex efflux pumps genes among the XDR and PDR isolates of *P. aeruginosa* obtained from various clinical specimens in Diyala Province hospitals. Therefore, the detection of Mex pumps positive *P. aeruginosa* isolates in this study indicates importance of strengthening surveillance to prevent the nosocomial infection and dissemination of this genes in Diyala.

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