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ORIGINAL ARTICLE

Detection of Efflux Pumps Overexpression in Flouroquinolone Resistant *Pseudomonas aeruginosa*

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ABSTRACT

Background: *P. aeruginosa exhibits several efflux pump systems that allow it to be resistant to several antimicrobial agents. Phenylalanine arginyl β-naphthylamide (PAβN) is an Efflux pump inhibitor (EPI) that can inhibit several multidrug efflux pumps.* ***Objective:*** *This study aimed to detect the efflux pump activity in FQ resistant P. aeruginosa, and to investigate the role of PAβN on FQ resistance.* ***Methodology:*** *P. aeruginosa isolates were subjected to antibiotic susceptibility testing by disc diffusion and those resistant to ciprofloxacin and levofloxacin were subjected to MIC detection before and after addition of PAβN. Also reverse transcription RT PCR was done for detection of mexA, mexC, mexE and mexX genes overexpression in the FQ resistant strains.* ***Results:*** *After the addition of PAβN, 95.9% and 94.5% of the isolates showed reduction in MICs of ciprofloxacin and levofloxacin respectively, 8.20% and 27.4% of the isolates restored susceptibility to those drugs respectively and 8.20% reverted to levofloxacin intermediate breakpoint. MexE was the most common efflux pump overexpressed, followed by mexX and mexA. When using EPI 96.8% and 95.3% of the isolates showed both overexpression and reduction of ciprofloxacin and levofloxacin MIC respectively. Using a cut- off point of ≥4 fold reduction in levofloxacin MIC discriminates efflux pump overexpressing from non-overexpressing isolates, (sensitivity of 70%, specificity of 67%).* ***Conclusion:*** *Efflux pump mediated resistance is an important mechanism contributing to multidrug resistance in P.aeruginosa as proven phenotypically and genotypically, with mexE being the most commonly expressed. The addition of PAβN to levofloxacin could be used as a phenotypic test for detection of efflux pump overexpressing isolates and may be effective to restore the levofloxacin susceptibility.*

INTRODUCTION

The undiscriminating use of antibiotics and the increasing emergence of resistance has exhausted the research in this field, with a declining rate of discovery for new antibiotics unable to counteract the escalation in resistance. Fluoroquinolones (FQs) until lately provided a broad-spectrum option for gram-negative infections. Unfortunately FQs resistance is on the rise, particularly for *P. aeruginosa* , known for causing various type of infections whenever the normal physiological function is disturbed particularly in hospitalized patients [1].

All recognized mechanisms of antibiotic resistance can be displayed by *P.aeruginosa* (intrinsic, acquired, and adaptive); sometimes all within the same isolate. Giving rise to infections caused by multidrug-resistant (MDR) *P.aeruginosa*, with a direct link to increased morbidity, mortality, increased length of hospital stay and increased hospital costs[2,3].

P.aeruginosas intrinsic resistance is due to the decreased outer membrane permeability, the natural occurrence of an inducible chromosomal β-lactamase, Amp C and the constitutive expression of membrane efflux (Mex) pumps[4]. Twelve potential efflux systems of the mex family have been identified in *P.aeruginosa* genome, 4 multicomponent MDR RND efflux pumps, mexAB-OprM, mexCD-OprJ, mexXY-OprM and mexEF-OprN are the best characterized as antibiotic transporters in this organism. Some are considered a major source of MDR since they are constitutively expressed, and can eject a wide range of antibiotics[5]. Moreover a single organism can show overexpression of more than one pump. This clarifies that some organisms were resistant to newly initiated classes of antibiotics although they were not previously exposed to them. Adding to this efflux mediated resistance may only be overexpressed during therapy which leads to treatment failure[6] .

So pumps overexpression is either constitutive (genotypic resistance), due to mutations in genes that normally down regulate their expression, or transient (phenotypic resistance) which occurs upon the exposure to inducers of the efflux pumps expression such as antibiotics which are able to induce overexpression and consequently resistance [7,8].

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From the study of efflux mechanisms acting on antibiotic-resistant strains of *P.aeruginosa*, Microcide and Daiichi Pharmaceuticals have produced a large family of peptidomimetics that exhibit Efflux Pump Inhibitor (EPI) properties. Among this family, the first identified EPI was Phenylalanine-arginyl-ß-naphthelamide (PaβN)[9] .

In the present study, we investigate the role of efflux pumps overexpression in FQ resistant *P.aeruginosa* strains phenotypically by using PAβN (EPI) and genotypically by measuring efflux pumps gene expression level.

METHODOLOGY

One hundred *P.aeruginosa* isolates from different clinical specimens of hospitalized patients in Alexandria Main University Hospital constituted the material of this study. This study was reviewed and approved by the ethical committee of the Faculty of Medicine, Alexandria University.

Suspected pseudomonas isolates were further identified and the susceptibility of all confirmed isolates were determined by disk diffusion technique[10] .

All *P.aeruginosa* isolates that were resistant to both ciprofloxacin and levofloxacin by disk diffusion were tested by broth microdilution technique to the 2 drugs to detect their minimal inhibitory concentration (MIC), using CLSI breakpoints (ciprofloxacin;S≤1 µg /ml, I=2 µg /ml, R≥4 µg /ml, Levofloxacin; S≤2 µg /ml, I=4 µg /ml, R≥8 µg /ml)[11] . *P.aeruginosa* ATCC 27853 was included and tested in each plate as a positive control.

Phenotypic Screening For the overexpression of efflux pump:

The ciprofloxacin and levofloxacin MIC for *P.aeruginosa* isolates were repeated as above but with the addition of the broad spectrum (EPI) (PAßN) (Sigma-Aldrich Ltd, USA) at a concentration of 20 µg /ml, with an additional control well of EPI and organism without antibiotic[12] .

Genotypic Detection of efflux Genes Overexpression:

Reverse transcription RT PCR was done for detection of mexA, mexC, mexE and mexX genes overexpression as representatives of the mexAB-OprM, mexCD-OprJ, mexEF-OprN and mexXY-OprM efflux pumps respectively[13] .

P. aeruginosa isolates were cultured at 37°C for 24 hours on MacConkey's agar plates prior to RNA extraction. A 1X10 [9] colonies of this plate were further subcultured in 4 ml of Luria-Bertani (LB) broth medium for 4 hours, to achieve late-logarithmic growth-phase, for high total RNA yield. Bacterial pellet was prepared by cold centrifugation of 1 ml of the cultured LB broth at 11.000 x g for 5 minutes and used directly for total RNA extraction.

RNA Extraction was done using the ISOLATE II RNA Mini kit (Bioline Ltd, UK) according to the manufacturer's instructions and prior to reverse transcription (RT)-PCR. Nanodrop was used to assess RNA concentration, where an optical density OD260/OD280 ratio between 1.8 and 2.1 indicated that RNA was of reasonable purity. The eluted RNA was stored at -70°C.

Reverse transcription and Quantitative Real-Time PCR

- MexA, mexC, mexE and mexX genes expression was detected using primers in table 1 to relatively quantify these genes against a control reference strain *P. aeruginosa* PAO1. The gene expression was normalized to that of the housekeeping gene rpsl[11,12].
- Using SYBR Green technology (SensiFASTTM SYBR No-ROX One-Step Kit, Bioline Ltd, UK). PCR reaction was performed at a final volume of 20 µl (table 2). The reaction was carried out using Rotor-Gene Q (Qiagen) Real-Time PCR (Corbett Research, Sydney, Australia; Model RG 3000), according to the following conditions; Initial Reverse transcription at 45°C for 10 minutes, then denaturation at 95°C for 10 minutes, followed by 40 cycles of (5 seconds at 95°C,10 seconds at 60°C,5 seconds at 72°C).

Table 1: Nucleotide sequence of primers used in the study

Gene	Primer	Sequence(5'-3')
mexA	Forward	GGCGACAACGCGGCGAAGG
	Reverse	CCTTCTGCTTGACGCCTTCCTGC
mexC	Forward	GCAATAGGAAGGATCGGGGCGTTGG
	Reverse	CCTCCACCGGCAACACCATTTCG
mexE	Forward	TCATCCCACTTCTCCTGGCGCTACC
	Reverse	CGTCCCACTCGTTCAGCGGTTGTTCGATG
mexX	Forward	AATCGAGGGACACCCATGCACATCC
	Reverse	CCCAGCAGGAATAGGGCGACCAG
Rpsl	Forward	GCAAGCGCATGGTCGACAAGA
	Reverse	CGCTGTGCTCTTGCAGGTTGTGA

Table 2: Volumes of each component per reaction of Real time PCR

Reagent	Volume	Final concentration
2x SensiFAST™ SYBR No-ROX One-Step Mix	10µl	1x
10µM Forward Primer	0.8µl *	400nM
10µM Reverse Primer	0.8µl *	400nM
Reverse transcriptase	0.2µl	-
RiboSafe RNase Inhibitor	0.4µl	-
H2O	up to 16µl	
Template	4µl	
20µl Final volume		

*Primer concentration: 10 picomoles/µl

Gene expression analysis was done by relative quantitation of mRNA from each gene of interest determined by the comparative threshold (CT). Each gene Expression was normalized against the rpsl housekeeping gene of the same strain. Data were conveyed as the relative expression of mex genes compared to the same genes in the PAO1 reference strain which was assigned a value of 1.0 (figure 1) [14] .

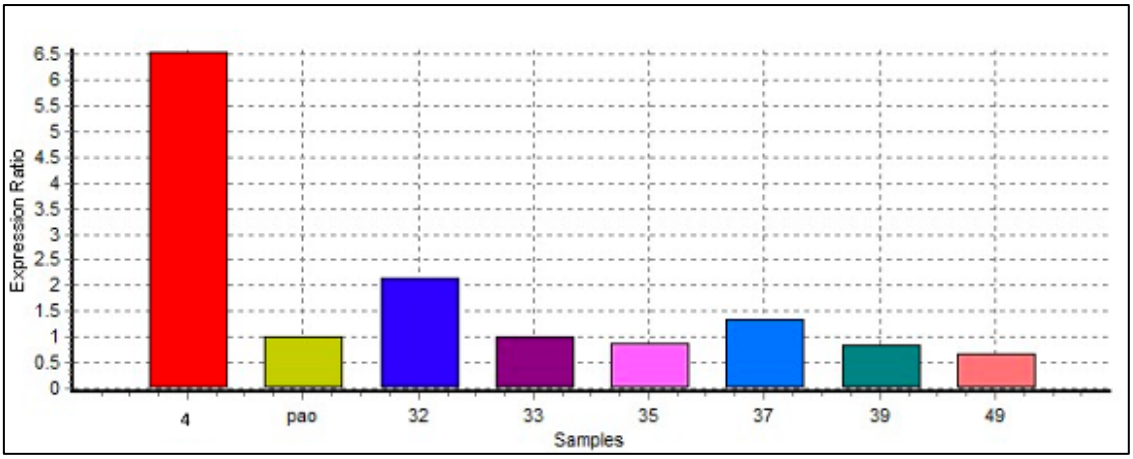

Fig. 1: Is an example of gene expression graph generated by Rotor Gene. The graph shows the expression ratio between the reference strain PAO1 and the tested strain regarding the gene of interest, which was mexE in this graph. Strain 4 had the highest expression ratio, followed by strain 32 and 37, while strains 35, 39, 49 showed under-expressions

The equation 2-ΔΔCT, where ΔΔCT = ΔCT-target - ΔCT-reference , permitted relative quantitation for the levels of gene expression between the target and the reference PAO1strain[15].

Melting curve analysis was used to assess the quality of the results : The presence of a single peak signified the occurrence of amplification in the target sequence alone and indicated that the data was safe for interpretation[16] .

RESULTS

Our 100 *P.aeruginosa* strains were isolated from different clinical samples and were all found to be MDR. Figure (2) shows the percentage of resistance to each antibiotic tested.

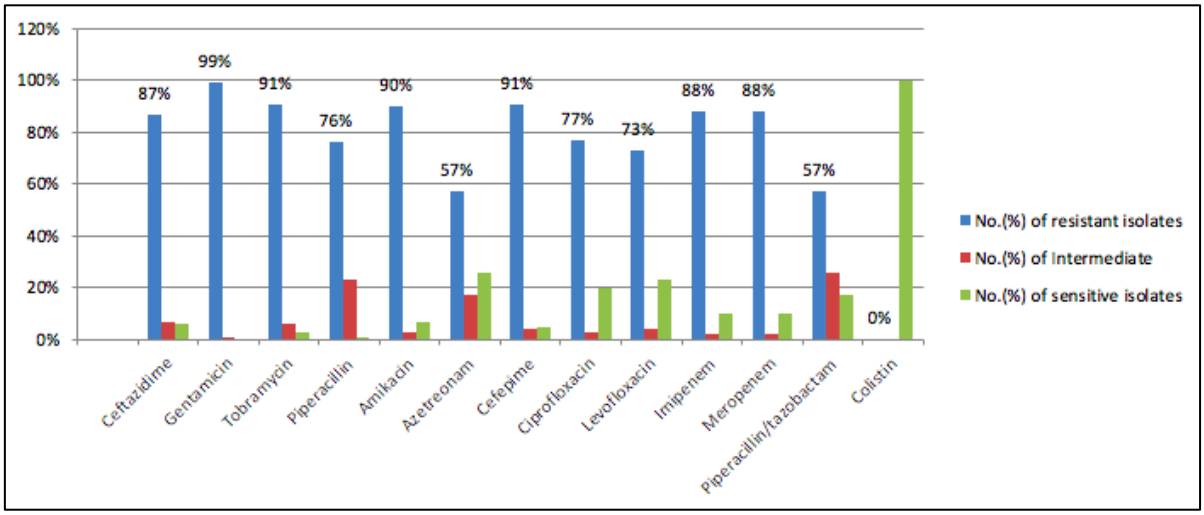

Fig. 2: The antimicrobial susceptibility pattern of the 100 P.aeruginosa isolates to different antimicrobial agents by disk diffusion technique

Out of the 100 isolates, 73 ciprofloxacin resistant strains were also found resistant to levofloxacin. Forty two (57.5%) of these shared the same antibiotic resistance profile, where they were resistant to all tested antibiotics except colistin, and (13/ 73, 17.8%) shared a nearly similar profile except, they were also sensitive to aztreonam.

Regarding the effect of (20µg/ml) PAβN (EPI) as a phenotypic test on the 73 FQs resistant isolates, seventy (70/73, 95.9%) and (69/73, 94.5%) of the isolates showed a reduction in MIC of ciprofloxacin and levofloxacin respectively. Out of which, (64/73, 87.7%) and (43/73,58.9%) of these isolates showed reduction in MIC of both ciprofloxacin and levofloxacin but still above the susceptible breakpoint (table 3).

Table 3: Role of PAβN in reduction of MIC of ciprofloxacin and levofloxacin on resistant isolates of *P.aeruginosa*

MIC reduction	Isolates tested for CIP MIC		Isolates tested for LEV MIC	
	No.	%	No.	%
No MIC reduction	3	4.1%	4	5.5%
MIC reduction above susceptible breakpoint	64	87.7%	43	58.9%
MIC reduction at or below susceptible breakpoint	6	8.2%	20	27.4%
MIC reduction to intermediate breakpoint	0	0%	6	8.20%
Total no. Of isolates	73	100%	73	100%
P value	0.106		0.0335*	

* P-value < 0.05 by Chi-square test is significant

In the current study, (6/73, 8.2%) and (20/73, 27.4%) of the isolates reinstated their sensitivity to ciprofloxacin and levofloxacin (table 3) correspondingly after adding PAβN. In addition to (6/73,8.2%) isolates that reverted to the intermediate levofloxacin MIC breakpoint. The number of isolates that regained their levofloxacin susceptibility were found statistically significant. PAβN had no effect on the MIC of ciprofloxacin in only (3/73, 4.1%) of the isolates and on the levofloxacin MIC in (4/73, 5.5%) of the isolates (table 3). Tables 4 and 5 show the ciprofloxacin and levofloxacin MIC fold reduction for the isolates tested.

Also the efflux pump genes were overexpressed in most of the tested isolates (65/73, 89.1%). The resistant isolates overexpressed one or more of the *mex* genes. Only (8/73, 10.9%) of isolates showed no overexpression of any of the four tested genes. *MexE* (51/73, 69.8%) was the most common efflux pump overexpressed among the resistant *P.aeruginosa* isolates, followed by *mexX* (49/73, 67.1%), and *mexA* (43/73, 58.9%) which was the highest singly overexpressed EP gene (6/73, 8.2%), whereas *mexC* (39/73,53.4%) was the least overexpressed. Twenty (20/73, 27.4%) of the isolates overexpressed all four tested RND efflux pump genes, followed by the

combination of *mexC+ mexE+ mexX* in (12/73, 16.4%) of the isolates.

Comparing both phenotypic and genotypic methods for detection of efflux pump, the current study showed that 62 out of the 64 isolates that showed genes of overexpression displayed reduction of ciprofloxacin MIC after the addition of PAβN (96.8%) and (61/64, 95.3%) showed reduction of levofloxacin MIC with PAβN. Whereas 9 *P.aeruginosa* isolates showed no overexpression of any of the four tested efflux pumps; One isolate (1/9,11.2%) showed no reduction in MICs and (8/9, 88.8%) isolates showed reduction in both MICs after the addition of PAβN. On the other hand, (2/64, 3.2%) and (3/64,4.7%) exhibited an overexpression of one or more efflux genes as detected by RT PCR although they showed no reduction of ciprofloxacin and levofloxacin MICs respectively.

To evaluate the PAβN as a phenotypic method for detection of efflux pump overexpression in aspect of sensitivity, specificity and accuracy through the detection of a cutoff point that confirms an isolate as efflux pump overexpressing a roc curve was performed. The roc curve revealed that the addition of PAβN to levofloxacin can significantly discriminate efflux pump overexpressing from non-overexpressing isolates. It has fair diagnostic performance at a cut-off point of ≥4 fold reduction in MIC with sensitivity of 70% and specificity of 67%. On the other hand ciprofloxacin with PAβN cannot significantly discriminate between efflux pump overexpressing and non-overexpressing isolates.

Table 4: Distribution of the FQ resistant *P.aeruginosa* isolates according to MIC Fold changes of ciprofloxacin after the addition of PAβN:

MIC (µg/ml)	No change	1-fold	2-fold	3-fold	4-fold	5-fold	6-fold	7-fold	Isolates with indicated MIC
≥512µg/ml	1		2	9	7	1			20 (27.3%)
256µg/ml		1	10	6	3	1			21 (28.7%)
128µg/ml		6	5	2					13 (17.8%)
64 µg/ml	1	2	1				1*	1*	6 (8.2%)
32 µg/ml		4		4			3*		11 (15%)
16µg/ml	1				1*				2 (3%)
Isolates with indicated fold reduction	3 4.1%	13 17.8%	18 24.7%	21 28.8%	11 15%	2 2.7%	4 5.5%	1 1.4%	73 (100%)

*Isolates reverting to susceptible breakpoint

Table 5: Distribution of the FQ resistant *P.aeruginosa* isolates according to MIC Fold changes of levofloxacin after the addition of PAβN:

MIC (µg/ml)	No change	1-fold	2-fold	3-fold	4-fold	5-fold	6-fold	7-fold	8-fold	9-fold	Isolates with indicated MIC
≥512µg/ml			2	2	6				4*	1*	15(20.5%)
256µg/ml			3	7	1	1	2*				14(19.2%)
128µg/ml			6	3		4*	2*	1*			16(21.9%)
64 µg/ml	1	2	2	1		1*					7(9.6%)
32 µg/ml	2	6	1		2*	6*	1*				18(24.7%)
16 µg/ml	1			1*	1*						3(4.1%)
Isolates with indicated fold reduction	4 5.5%	8 10.9%	14 19.2%	14 19.2%	10 13.7%	12 16.4%	5 6.8%	1 1.4%	4 5.5%	1 1.4%	**73 (100%)**

*Isolates reverting to susceptible breakpoint

DISCUSSION

The high resistance of *P.aeruginosa* in the current study has been noticed by others in Egypt, which necessitates the development of a national antibiotic policy as global studies show much lower MDR rates[17].

Considering each group of antibiotics gentamicin, tobramycin and amikacin resistance (99 %,91% and 90%) were higher than that reported by other studies locally and elsewhere[15,16] .

As for β-lactams particularly resistance against third and fourth generation cephalosporins in Egypt over the past decade from 2007 through 2012 and up to 2018 reveals an alarming ascending pattern, as observed in our setting.[14,15,17] Similarly Carbapenem resistance is also becoming a challenge limiting the therapeutic options, on the other hand all of the tested isolates in our study showed no resistance to colistin, making colistin a precious effective antibiotic for treatment of serious *P.aeruginosa* infections in our setting. This was followed by aztreonam and piperacillin/tazobactam with resistance rates (57%) similar to other reports [18,19] .

Forty two (57.5%) of our FQ resistant strains shared the same antibiotic resistance profile, indicating that FQs resistant isolates may share common resistance mechanism with other antibiotics, i.e. FQ resistance could be in synchrony with resistance to other antipseudomonal agents through overexpression of FQ mediated multidrug efflux pumps, with broad substrate specificity for the extrusion of many antibiotic classes. On the other hand, the 27 *P.aeruginosa* isolates that were susceptible to ciprofloxacin and/or levofloxacin showed 18 different antibiotic resistance profiles.

FQs Resistance is either due to gene mutations for drug targets or active efflux. The favored resistance mechanism by *P. aeruginosa* on first exposure to FQs is efflux. In case of re-exposure, consequent mutations are expected, for example in the gyrase gene up stepping the resistance level [20,21].

The inhibition of the efflux pumps is expected to decrease the level of intrinsic drug resistance, significantly reverse acquired resistance and hopefully decrease the frequency of emergence of *P.aeruginosa* mutants highly resistant to FQs. Inhibitors of efflux pumps if combined with the antibiotic is a promising approach towards the improvement of the clinical efficacies of such antibiotics. EPI may also be used as phenotypic tests to recognize the presence of acquired multidrug resistance due to efflux pump overexpression in a laboratory setting [22].

Regarding the effect of (20µg/ml) PAβN (EPI) as a phenotypic test on the 73 FQs resistant isolates, our results were higher than what was claimed elsewhere [18]. The number of isolates that regained their levofloxacin susceptibility were found statistically significant. However it was stated that increasing the concentration of EPI would have more effect on FQs MICs due to altering membrane permeability rather than inhibition of efflux pumps only [23,24].

Our findings indicate that levofloxacin with PAβN was a better combination for reversing FQ resistance in our strains. Indicating that EPI action is not only determined by the efflux pump it targets but also on the antimicrobial it supplements. Consequently, the proposal of an EPI of equal efficiency on all (RND) efflux pumps will be difficult.

The percentage of our *P. aeruginosa* isolates that were not affected by the inhibitor was low when compared to others [25] .

MexE was the most overexpressed efflux pump gene in most of the tested isolates, followed by *mexX* and *mexA*, others who reported *mexA* or *mexX* to be the most frequent genes overexpressed [26] .

The variation in the occurrence of different efflux pumps could be explained by the different antibiotics use in each setting. The antibiotic used may interfere with the selection of the most overexpressed efflux system, particularly for the constitutively expressed pumps MexAB-OprM and MexXY-OprM. A pump is overexpressed in *P. aeruginosa* isolates with mutations in regulator genes. These mutants can be selected during treatment with the pump's substrate antibiotic [22] .

During the present study, no antibiotic stewardship was followed in our hospital, any combination of fluoroquinolones, penicillins, cephalosporins and carbapenems could be used for treatment. This may explain the nearly close overexpression percentage of all four tested pumps.

Nine FQ resistant *P.aeruginosa* isolates showed no overexpression of any of the four tested efflux pumps; One isolate showed no reduction in MICs, this was also a finding by others who attributed this to the possibility of the inhibitor increasing the outer membrane permeability of Gram-negative bacteria, and thus reducing the MICs or there could be other efflux pumps that were inhibited by the PAβN [25] .

In the present study (3.2%-4.7%) exhibited an overexpression of one or more efflux genes with no reduction of ciprofloxacin and levofloxacin MICs respectively. This was less than what was reported [25] . One factor possibly contributing to this difference in evaluating the PAβN as a phenotypic test is the breakpoints used in MIC fold reductions to confirm that the isolates showed efflux pumps overexpression.

A roc curve was performed to evaluate the PAβN as a phenotypic method for detection of efflux pump overexpression in aspect of sensitivity, specificity and accuracy through the detection of a cutoff point that confirms an isolate as efflux pump overexpressing. The roc curve revealed that the addition of PAβN to levofloxacin can significantly discriminate efflux pump

overexpressing from non-overexpressing isolates, at a cut-off point of ≥4 fold reduction in MIC(sensitivity of 70% and specificity of 67%). The four fold reduction in MIC cutoff was used in different studies [24,26] .

CONCLUSION

To conclude, our findings suggest that Efflux pump mediated resistance is an important mechanism contributing to multidrug resistance in clinical isolates of *P.aeruginosa*' with mexE being the most commonly expressed gene. The possibility of addition of PAβN to levofloxacin to be used as a phenotypic test for detection of efflux pump overexpressing isolates is promising and should also be further investigated as an effective method to restore the levofloxacin susceptibility.

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- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

Authors contribution:

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by May Mohamed Elghamrawi, Hadir Ahmed Said Okasha. The first draft of the manuscript was written by Hadir Ahmed Said Okasha. All authors read and approved the final manuscript.

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