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Original Research Article

# Detection of Mex Efflux Pumps in *Pseudomonas aeruginosa* using a Multiplex Phenotypic and Genotypic Procedure in an Iranian Referral Hospital

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**ABSTRACT**

**Background:** The multidrug resistance phenotype of *Pseudomonas aeruginosa* is largely attributable to expression of Mex efflux pumps. Detection of such pumps expression in different clinical setting would help to select the best antibiotic treatment of choice. **Materials and Methods:** A multiplex phenotypic and genotypic method was applied for the differential recognition of resistance mediated by the four major transporters, including MexAB-OprM, MexCD-OprJ, MexEF-OprN, andMexXY-OprM. The multiplex approach was performed on 40 selected clinical isolates. Minimum Inhibitory Concentration (MIC) method was respectively used for phenotypic detection using reporter antibiotics Carbenicillin,Erythromycin,Norfloxacin,Imipenem and gentamicin. Genotype was detected using semi-quantitative and quantitative competitive real-time PCR of mex C and mex Eandmex A and mex X, respectively. Both methods were relevant with good levels of overexpression. **Results:** There were compare and research between phenotypic and genotypic methods in control strains for allmentioned pumps. Among clinical isolates, similarity was observed in 28 out of 40 strains for MexAB-OprM and MexCD-OprJ, and in 32 out of 40 strains for MexXY-OprM and MexEF-OprM. **Conclusion:** Our results validate the multiplex phenotypic and genotypic as a suitable methodsfor the diagnosis of resistance mediated by four Mex efflux pumps in *P. aeruginosa* and consequently application of the correct antibiotic.

## INTRODUCTION

*Pseudomonas aeruginosa* is one of the major nosocomial pathogens worldwide. Nosocomial infections caused by this organism are often hard to treat. This is because of both the intrinsic resistance of the species, and its remarkable ability to acquire extra resistance mechanisms to multiple groups of antimicrobial agents. [1,2] *Pseudomonas aeruginosa* is intrinsically resistant to many antimicrobial agents. This intrinsic resistance can be ascribed to synergy between an outer membrane with low permeability [3] and other contributing mechanisms, most especially drug efflux. [4,5] Genome analysis has displayed that *P. aeruginosa* codifies as many as 12 possible efflux systems of the RND family alone. [6] Of these, only four,

MexAB-OprM, [7]MexCD-OprJ, [8] MexEF-OprN, [9] and MexXY, [10,11] have been determined. In addition, they can also be used for genotyping as performed to determine pattern of antibiotic resistance or microbial fingerprinting. The semi-quantitative reverse transcription PCR (RT-PCR) [12-15] or quantitative real-time PCR [16-19] have been successfully used for detecting the expression of efflux pumps in *P. aeruginosa*. Previous study demonstrated a combined phenotypic and genotypic method for the detection of Mex efflux pumps in *Pseudomonas aeruginosa*, although the method was applied for 7 clinical samples.[20] In this study, a multiplex phenotypic and genotypic method was extended for the detection of resistance by Mex-mediated efflux in *P. aeruginosa*, focusing on MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM. A study on the phenotypic method has already been done, and the results re-used to overlap and increase precision with the genotype method in this study.

# MATERIALS AND METHODS

## Bacterial strains, drug resistance testing

Forty clinical isolations of *Pseudomonas aeruginosa* were collected between 2014 and 2015 at Alborz University-affiliated hospitals (Tehran, Varamin and Alborz state) in north of Iran listed in table1. All bacterial isolates were identified in the Microbiology Laboratory instead using CMC standard biochemical identification methods. *Pseudomonas aeruginosa* PAO1, which genome has entirely been sequenced, was used as the reference wild-type strain throughout the study. Reference strains with prove resistance by efflux pumps suggested as: PT629, JFL30 (MexAB-OprM); [21,22] EryR, JFL28(MexCD-OprJ); [21,23] PAO7H, JFL05(MexEF-OprN); [21,24] and MutGR1, JFL10(MexXY-OprM). [21,25]

These isolates were selected based on antibiotic resistance to carbenicillin (100 µg), erythromycin (15 µg), gentamicin (30 µg), imipenem (10 µg), and norfloxacin (5 µg) using disk diffusion method. 13 All antibiotic discs were purchased from Mast Company (United Kingdom).

The antibiotics of MICs: Gentamicin, imipenem, and erythromycin were obtained from Mast (London, UK) and PaβN, carbenicillin and orfloxacin were acquired from Sigma-Aldrich (St Louis, MO, USA). All other reagents were obtained from either Merck AG (Darmstadt, Germany) or Sigma-Aldrich. [26]

## Phenotypic analysis

The presented antibiotics of the Mex efflux pumps for phenotypic markers were: Carbenicillin (MexAB-OprM) (4 mg/L increase in the 8–32 mg/L), erythromycin (MexCD-OprJ), gentamicin (MexXY-OprM) (0.1 mg/L increase in the 0.25–1 mg/L) and, norfloxacin and imipenem (MexEF-OprN). [22,23,25,27,28] MICs were determined by using two-fold both microdilution method in the presence and absence [29] of a broad spectrum inhibitor of Mex pumps including phe-arg- β-naphthylamide (Pa β N), at the concentration of 50 mg/L (Sigma Aldrich, St. Louis, MO, USA). The MICs neither increase the susceptibility of reference strains to the reporter antibiotics nor it impacts the growth continues. It should be noted that erythromycin has been defined as a potential substrate of MexJK and MexVW. [30]

## Genotypic analysis

## Isolation of total RNA and synthesis of cDNA for RT-PCR

The bacteria were cultured overnight at 37°C and then total RNA was extracted using an RNA extraction kit (Fermentas, Lithuania), according to the manufacturers' instructions.The quality and quantity of RNA concentrations were monitored using Nano Drop 2000c (Eppendorf, Germany). RNA was reversely transcribed using the cDNA Synthesis kit (Fermentas, Lithuania), according to the manufacturer's instructions. The sequences of the genes-specific primers are listed in Table 3.

## Real time PCR reaction

Detection of mexA, mexX, mexC and mexE genes was performed by real-time PCR, quantitative competitive RT-PCR (QC-RT-PCR), andsemi-quantitative RT-PCR.

Real-time PCR was performed for mexA and mexX using an ABI Step One Detection System (Applied Biosystems, Warrington, United Kingdom) and SYBR®Premix Ex Taq™II Real Time PCR Master Mix (Takara, Japan), according to the manufacturer's instructions. Each reaction

contained 5µl master mix, 100 nM specific primers for rpsL, mexA, and mexX plus 1µl template cDNA. Thermocycler conditions included an initial denaturation at 95°C for 15min, followed by 40 cycles of 95°C for 15s and 64°C for 15s and 72°C for 20s. The rpsL gene used as was chosen as an internal control against which mRNA expression of the target gene was normalized.[16,17,31,32] The threshold cycle of samples was always within the range of standards (R2 ≥1).

The quantitative competitive RT-PCR (QC-RT-PCR) for mexA,mexX was performed using standard techniques with the following specific conditions. Internal competitor DNAs were synthesized by PCR using 0.6 mM primers (mexA-1 and mexA-2 or mexX-1 and mexX-2), 10 ng template DNA, 1.5 M betaine (Roche Molecular Biochemicals, Mannheim, Germany), 1.25 mM mixed deoxynucleoside triphosphates (Fermentas GmbH, St Leon-Rot, Germany) and 2 U of Taq DNA Polymerase in the corresponding buffer (1-dilution; Biotools, B & M Labs S.A, Madrid, Spain).[20,33-35] The quantitative competitive RT-PCR conditions for mexA amplification consisted of 15 min denaturation at 95°C, 30 cycles of 45s at 95°C, 45s at 64°C and 45s at 72°C. The condition for mexX amplification consisted of 30 cycles of 1 min at 95°C, 1 min at 64°C and 1min at 72°C, and a final elongation at 72°C for 10 min.

Expression of mexC and mexE was evaluated using semi-quantitative RT-PCR. The reaction consisted of thirty picomoles of each primer (listed in Table 3), witha 50-µlfinal volume. The reaction was then subjected to a 30-min incubation at 50°C followed by 15min at 95°C and 40 cycles of 30s at 94°C, 30s at 55°C, 30s at 72°C, and terminated with 10min at 72°C. For a positive control, mexA at a basal level in all strains was amplified. [4,15]

In the phenotypic analysis, efflux was considered as likely when the MIC of a given strain was increased by 1-dilution; and efflux was considered as possible if only the MIC remained high after addition of PAβN and compared with the wild strain. In the genotypic analysis, MexAB-OprM and MexXY-OprM-based efflux was considered possible when mexAand mexX were over expressed ≥3-fold, comparing to the wild strain. In addition, when mexC and mexE were expressed, the MexCD-OprJ and MexEF-OprN efflux were assumed likely. [4,35]

Similarities between these methods was considered as complete when both methods showed the possible presence of the related efflux mechanism. The efflux was assumed when both of the phenotypic and genotypic methods complete each other. Otherwise, the efflux was assumed unlikely when the phenotypic and genotypic methods had differences.

# RESULTS

Forty clinical isolates of *Pseudomonas aeruginosa* were collected from different sections of hospital and clinical samples. The majority of these isolates were collected from patients hospitalized in intensive care units PICU (n=6, 15%), NICU (n=4, 10%), followed by emergency ward (n=3, 7.5%), nephrology(n=5, 12.5%), surgery (n=6, 15%), neurology (n=5, 12.5%), urology (n=6, 15%), and infectious diseases ward (n=5,12.5%).

These investigated samples were assigned the following names: D1, E1, G3, H2, L2, S3, A2, G4, I, K1, P2, A1, E2, G1, H1 ,M1, S1, C2, D2, O1, Q, S2, U1, L1, N, O2, T1, W, B, C1, D3, J, F, M2, K2, T2, P1, P3, R1, R2.

The MIC values were measured with 50 mg/l, PAβN as efflux inhibitor, as shown in Table 2. The MICs of carbenicillin and gentamicin decreased 37% (≠15out of 40) and 22% (≠9 out of 40) by addition of PAβN in PAO1 in fundamental expression ofMexAB-OprM and MexXY-OprM.[36,37] The MIC of erythromycin was markedly decreased in PAO1 by addition of PAβN, suggesting the presence of another PAβN-inhabitable efflux transporter in this strains. Previously erythromycin has been described as a potential substrate of MexJK and MexVW in the 40 clinical isolates. The MICs for carbenicillin in dilutions higher than+1 were observed in 85% of the cases (34 out of 40). However, MICs for erythromycin, norfloxacin, imipenem, and gentamicin were observed in 40% (16 out of 40), 85% (34 out of 40), 55% (22 out of 40), and 40% (16 out of 40) of the cases, respectively. In the presence of PAβN, MexAB-OprM in 28 (70%) samples; MexCD-OprJ in 17 (42%) samples; MexEF-OprN in 22 (55%) samples, and MexXY-OprM in 17 (42%) samples were active.

Six isolates (B, D3, F, K2, P1, and R1) had not significant by increase the amount of MIC.

MexA and mexX are fundamentally over expressed at essential and variable levels in wild-type intrinsic resistant strains. [4,38] Therefore, the expression of these genes was evaluated for genotypic analysis. MexA and mexX were over expressed by 3 to 8 times in reference strains. Such an expression was observed in 34 clinical isolates for mexA and 17 samples for mexX, as shown in Table 5. The comparison of QC-RT-PCR results with real-time RT-PCR results revealed 100% agreement for the detection of mexA and mexX in clinical isolates.

Expression of mexC and mexE were only determined using semi-quantitative analysis method. This is because, these genes are strictly down-regulated in wild-type strains and are highly expressed in resistant strains.

Expressions of MexC and mexE were markedly raised in 44% of reference strains. The expression of the corresponding genes in clinical isolates is shown in Table 5. Phenotypic analysis of isolates was initially performed based on over expression of mexA or mexX which was observed in isolates named D1, E1, G3, H2, L2, S3, A2, G4, I, K1, P2, A1, E2, G1, H1 ,M1, S1, C2, D2, O1, Q, S2, U1, L1, N, O2, T1, W. Further analysis based on over expression of MexAB-OprM was performed and observed in isolates A1, E2, G1, H1, M1, S1, C2, D2, O1, Q, S2, U1, L1, N, O2, T1, and W. Analysis based on MexXY-OprM was also performed and observed in isolates D1, E1 ,G3, H2, L2, S3, A2, G4, I, K1, P2) samples. In addition, six of the isolates including B, D3, F, K2, P1, R1, showed a low level of resistance to gentamicin with 4-fold decrease in MIC with PAβN.

Comparison of the phenotypic and the genotypic methods of the reference strains and the 40 clinical isolates were made, and are reflected in Table 5. A remarkable correlation was found between the two methods in reference strains. It means phenotypic display weaker role of efflux than indicate from genotypic data.

MexEF-OprN and MexXY-OprM were greatly related in the clinical strains: 34 strains showed complete similarity, 6 strains showed partial similarity, and no strain was difference. Result with MexAB-OprM was partially related in the second step: 28 strains had complete similarity, 12 strains showed partial similarity, and no strain was difference. Nevertheless, result with MexCD-OprJ showed slight correlation, where 29 strains were completely similarity, 5 strains were imperceptible similarity, and 6 strains were difference. The phenotypic method for the difference strains is either fully applicable or partially applicable to detect the presence of an efflux-mediated resistance. However, the semi-quantitative genotypic method is not applicable for the difference strains.

## DISCUSSION

This study offers a concomitant phenotypic and genotypic evaluation and the specific diagnosis of resistance mediated by four Mex efflux pumps in *P. aeruginosa*. In wild-type strains MexAB-OprM and MexXY-OprM are expressed at a fundamental level, contributing to the low intrinsic susceptibility of *P. aeruginosa* to the corresponding antibiotics, but are upregulated in resistant strains. [7,25,33,36,37] Two efflux pump, MexCD-OprJ and MexEF-OprN are not found in standard conditions ofwild-type *P. aeruginosa* culture, but their expression is up-regulated in resistant bacteria. [9,14,39] Our result demonstrated that lower concentrations suffice to reverse resistance to all substrates of a given pump, which is inconsistent with the previous study. [20] However, our results were consistent with the previous report indicating that large concentrations could express various efflux mechanisms for clinical isolates.[40-42]

The special detection of efflux pumps has long been dependent on western blot [9,24,36,39] or northern blot analysis. [43] However, these methods cannot simply be performed in the clinical laboratory. Because of speed and potentially high level of specificity, the nucleic acid-based diagnostic approaches are slowly replacing or complementing culture-based biochemical and immunological evaluations in the usual microbiology laboratories. [31]

The phenotypic method provides a first level of differentiation among these four Mex efflux pumps. The difficult explanation of phenotypic data continues to exist with clinical strains. This is because of the expression of resistance mechanisms other than efflux. Therefore, in this study we used two methods, which can be used substitute for each other, one is real-time PCR and the other is QC-RT-PCR assays for detecting over expression of

constitutively expressed pumps in a quantitative manner.

We did not apply real-time PCR and QC-RT-PCR methods for finding of the MexCD-OprJ and MexEF-OprN pump genes. However, the prior studies have consistently shown that the expression of mexC or mexE is seriously had low adjustedin wild-type strains. [8,9,12,16]

The results of genotypic methods do not prepare information needs on the final expression of the genes and the activities.[17] We have shown that the fundamental expression level of mexAis much more than mexX; however, both efflux pumps are over expressed 2 to 10 times more in resistant strains. The more amount of MexAB-OprM compared to MexXY-OprM indicated that effective transport of the relevant precursors may be needed. [20,39]

In another study, mexX over-expression in clinical isolates was shown to be systematically related to mexA. [8] This may be based on the fact that MexXY uses OprM as a porinchannels whilethe regulator operon MexAB-OprM expression is under the control and had highly over-expression. [36,44]

The PAβN decrease the MIC of imipenem in MexEF-OprN reference strains, although it use as precursor of this pump. A transport sectional of imipenem by MexEF-OprN may be caused by potential reasons, or by not definedMex pump inhibitor via PAβN. The PAβN decreases the pump expression and increases the OprD porin expression on the usual adjuster. [8]

In this study the basis of the results of the phenotype method was previously presented in a separate article and we used here to upgrade and further our research.

The clinical isolates were found to express several efflux pumps at the same time leading to exclusively multidrug-resistant phenotypes intricate. In this study, some findings are not exactly similar to the ones mentioned by others, based on their methods, goals and aims. During our study in addition to the technical and potential clinical diagnostic methodology, some biological observations were also significant. This study may present different results in other Mex(es), although they are not mentioned and examined in this research. In this regard, a further study is warranted.

## CONCLUSION

The results indicate a great performance for the detection of efflux using suitable phenotypic and genotypic methods to correct recognition of resistance mechanisms in common and usual clinical microbiology organisms.

We believe that based on the results of this study, development and popularization of these methods in large number of strains; is possible. Based on epidemiological studies, the most dominant and common resistance mechanisms may help individual patients, understand rationalizing the choice of their prescribed antibiotic treatment and dosing, at hospital settings, and for aid in developing appropriate antibiotic prescribing policies and formularies.

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Table 2: MICs of the four antibiotics selected as markers for MexAB-OprM [carbenicillin(CAR)], MexCD-OprJ [erythromycin (ERY)], MexEF-OprN [norfloxacin (NOR), imipenem (IMP)] or MexXYOprM [gentamicin (GEN)] and tested in the absence or in the presence of the wide spectrum efflux inhibitor PAβN (50 mg/L) for Strains of *Pseudomonas aeruginosa* used in this study

| MIC (mg/L) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CAR | | ERY | | NOR | | IMP | | GEN | |
| Strains | Ref | -PAβN | +PAβN | -PAβN | +PAβN | -PAβN | +PAβN | -PAβN | +PAβN | -PAβN | +PAβN |
| Reference strains with demonstrated resistance by efflux (genotype) | | | | | | | | | | | |
| PAO1(wild type) | | 16 | 16 | 128 | 16 | 0.4 | 0.4 | 1 | 1 | 0.5 | 0.5 |
| PT629 (MexAB-OprM) | [22] | 256 | 16 | 128 | 16 | 0.8 | 0.4 | 1 | 1 | 0.5 | 0.5 |
| JFL30 (MexAB-OprM) | [21] | 256 | 32/16 | 128 | 16 | 0.8 | 0.4 | 1 | 1 | 0.5 | 0.5 |
| EryR (MexCD-OprJ) | [23] | 16 | 16 | 4096 | 16 | 0.8 | 0.4 | 1 | 1 | 0.5 | 0.5 |
| JFL28 (MexCD-OprJ) | [21] | 32 | 16 | 2048 | 16 | 0.8 | 0.4 | 1 | 1 | 0.5 | 0.5 |
| PAO7H (MexEF-OprN) | [24] | 16 | 16 | 64 | 16 | 3.2 | 0.2 | 4/8 | 1 | 0.5 | 0.5 |
| JFL05 (MexEF-OprN) | [21] | 16 | 16 | 128 | 16 | 6.4 | 0.4 | 4 | 1 | 0.5 | 0.5 |
| MutGR1 (MexXY-OprM) | [25] | 32 | 16 | 128 | 16 | 0.4 | 0.4 | 1 | 1 | 8 | 0.5 |
| JFL10 (MexXY-OprM) | [21] | 32 | 16 | 128 | 16 | 0.4 | 0.4 | 1 | 1 | 4 | 0.5 |
| Clinical isolates with resistance by efflux | | | | | | | | | | | |
| A1 | | 128 | 32 | 512 | 16 | 3.2 | 0.8 | 8 | 4 | 1 | 1 |
| A2 | | 128 | 32 | 256 | 16 | 0.4 | 0.4 | 1 | 1 | 16 | 0.5 |
| B | | 32 | 16 | 256 | 16 | 1.6 | 0.4 | 16 | 8 | 1 | 0.25 |
| C1 | | 256 | 256 | 512 | 16 | 12.8 | 3.2 | 8 | 2 | 0.5 | 0.5 |
| C2 | | 128 | 32 | 256 | 16 | 25.6 | 6.4 | 8 | 4 | 0.5 | 0.5 |
| D1 | | 4096 | 128 | 256 | 128 | 25.6 | 25.6 | 1 | 1 | 128 | 8 |
| D2 | | 128 | 32 | 256 | 16 | 25.6 | 6.4 | 8 | 4 | 0.5 | 0.5 |
| D3 | | 32 | 16 | 256 | 16 | 1.6 | 0.4 | 16 | 8 | 1 | 0.25 |

| E1 | 4096 | 128 | 256 | 128 | 25.6 | 25.6 | 1 | 1 | 128 | 8 |
|---|---|---|---|---|---|---|---|---|---|---|
| E2 | 128 | 32 | 512 | 16 | 3.2 | 0.8 | 8 | 4 | 1 | 1 |
| F | 32 | 16 | 256 | 16 | 1.6 | 0.4 | 16 | 8 | 1 | 0.25 |
| G1 | 128 | 32 | 512 | 16 | 3.2 | 0.8 | 8 | 4 | 1 | 1 |
| G3 | 4096 | 128 | 256 | 128 | 25.6 | 25.6 | 1 | 1 | 128 | 8 |
| G4 | 128 | 32 | 256 | 16 | 0.4 | 0.4 | 1 | 1 | 16 | 0.5 |
| H1 | 128 | 32 | 512 | 16 | 3.2 | 0. 8 | 8 | 4 | 1 | 1 |
| H2 | 4096 | 128 | 256 | 128 | 25.6 | 25.6 | 1 | 1 | 128 | 8 |
| I | 128 | 32 | 256 | 16 | 0.4 | 0.4 | 1 | 1 | 16 | 0. 5 |
| J | 256 | 256 | 512 | 16 | 12.8 | 3.2 | 8 | 2 | 0.5 | 0.5 |
| K1 | 128 | 32 | 256 | 16 | 0.4 | 0.4 | 1 | 1 | 16 | 0.5 |
| K2 | 32 | 16 | 256 | 16 | 1.6 | 0.4 | 16 | 8 | 1 | 0.25 |
| L1 | 128 | 32 | 512 | 64 | 25.6 | 25.6 | 2 | 2 | 128 | 128 |
| L2 | 4096 | 128 | 256 | 128 | 25.6 | 25.6 | 1 | 1 | 128 | 8 |
| M1 | 128 | 32 | 512 | 64 | 3.2 | 0.8 | 8 | 4 | 1 | 1 |
| M2 | 256 | 256 | 512 | 16 | 12.8 | 3.2 | 8 | 2 | 0.5 | 0. 5 |
| N | 128 | 32 | 512 | 64 | 25.6 | 25.6 | 2 | 2 | 128 | 128 |
| O1 | 128 | 32 | 256 | 16 | 25.6 | 6.4 | 8 | 4 | 0.5 | 0.5 |
| O2 | 128 | 32 | 512 | 64 | 25.6 | 25.6 | 2 | 2 | 128 | 128 |
| P1 | 32 | 16 | 256 | 16 | 1.6 | 0.4 | 16 | 8 | 1 | 0.25 |
| P2 | 128 | 32 | 256 | 16 | 0.4 | 0.4 | 1 | 1 | 16 | 0.5 |
| P3 | 256 | 256 | 512 | 16 | 12.8 | 3.2 | 8 | 2 | 0.5 | 0.5 |
| Q | 128 | 32 | 256 | 16 | 25.6 | 6.4 | 8 | 4 | 0.5 | 0.5 |
| R1 | 32 | 16 | 256 | 16 | 1.6 | 0.4 | 16 | 8 | 1 | 0.25 |
| R2 | 256 | 256 | 512 | 16 | 12.8 | 3.2 | 8 | 2 | 0.5 | 0.5 |

| S1 | 128 | 32 | 512 | 16 | 3.2 | 0.8 | 8 | 4 | 1 | 1 |
|---|---|---|---|---|---|---|---|---|---|---|
| S2 | 128 | 32 | 256 | 16 | 25.6 | 6.4 | 8 | 4 | 0.5 | 0.5 |
| S3 | 4096 | 128 | 256 | 128 | 25.6 | 25.6 | 1 | 1 | 128 | 8 |
| T1 | 128 | 32 | 512 | 64 | 25.6 | 25.6 | 2 | 2 | 128 | 128 |
| T2 | 256 | 256 | 512 | 16 | 12.8 | 3.2 | 8 | 2 | 0.5 | 0.5 |
| U1 | 128 | 32 | 256 | 16 | 25.6 | 6.4 | 8 | 4 | 0.5 | 0.5 |
| W | 128 | 32 | 512 | 64 | 25.6 | 25.6 | 2 | 2 | 128 | 128 |

Table 3: Sequences of primers used in this study

| Gene | primer | DNA sequence (5'- 3') | Size (bp) | Reference |
|---|---|---|---|---|
| *MexA* | F | *CCTGCTGGTCGCGATTTCGG* | 312 | [21] |
| | R | *CCAGCAGCTTGTAGCGCTGG* | | |
| *MexX* | F | *GCGATGCGGATTGCGGAACA* | 792 | |
| | R | *TGGTCGCCCTATTCCTGCTG* | | |
| *MexC* | F | *TTGGCTATGGCCATCGCGTT* | 371 | |
| | R | *ATCGAAGTCCTGCTGGCTGA* | | |
| *MexE* | F | *ATCCCACTTCTCCTGGCGCT* | 240 | |
| | R | *GGTCGCCTTTCTTCACCAGT* | | |
| *rpsL* | F | GCAAGCGCATGGTCGACAAGA | 201 | [16] |
| | R | CGCTGTGCTCTTGCAGGTTGTGA | | |

Table 4: The expression levels of *mexA and mexX* genes by QC-RT-PCR and real time PCR and the values determined by the two techniques.

| | *MexA* expression level | | | *MexX* expression level | | |
|---|---|---|---|---|---|---|
| Strain | QC-RT-PCR | The realtime PCR | QC-RT-PCR/ real time PCR | QC-RT-PCR | The realtime PCR | QC-RT-PCR/ realtime PCR |
| Reference strains | | | | | | |
| PAO1 | 1 | 1 | | 1 | 1 | |
| PT629 | 3.97±0.10 | 3.75±0.05 | 1.07 | ND | 0.20±0.02 | |
| JFL10 | ND | 1.12±0.17 | | 7.12±0.15 | 6.51±0.11 | 1.09 |
| JFL30 | 5.45±0.15 | 5.04±0.10 | 1.08 | ND | 2.12±0.01 | |
| MutGR1 | ND | 1.41±0.21 | | 5.12±0.20 | 5.58±0.40 | 0.89 |
| Clinical isolates | | | | | | |
| A1 | 5.34±0.26 | 5.07±0.35 | 1.03 | ND | 0.17±0.04 | |
| A2 | 5.76±0.29 | 5.84±0.23 | 0.9 | 3.71±0.15 | 3.42±0.27 | 1.04 |
| B | ND | 2.20±0.18 | | 4.67±0.20 | 5.11±0.7 | 0.82 |
| D1 | 7.15±0.17 | 6.62±0.32 | 1.08 | 8.15±0.15 | 8.59±0.17 | 0.95 |
| L1 | 9.56±0.07 | 9.37±0.14 | 1.02 | ND | 0.54±0.11 | |
| C2 | 7.51±0.02 | 6.48±0.12 | 1.14 | ND | 0.21±0.03 | |
| E2 | 4.45±45 | 4.38±0.20 | 1.01 | ND | 0.23±0.02 | |
| D2 | 5.17±0.45 | 4.55±0.37 | 1.14 | ND | 0.18±0.07 | |
| D3 | ND | 3.48±0.28 | | 6.13±0.24 | 6.21±0.36 | 0.96 |
| G4 | 4.23±0.22 | 4.40±0.45 | 0.89 | 7.54±0.27 | 7.45±0.20 | 1.02 |
| N | 7.64±0.17 | 7.58±0.22 | 1 | ND | 0.35±0.21 | |
| G1 | 6.32±0.12 | 6.12±0.20 | 1.01 | ND | 0.15±0.03 | |
| E1 | 5.74±0.12 | 5.27±0.20 | 1.07 | 7.14±0.21 | 7.29±0.49 | 0.94 |
| F | ND | 2.48±0.07 | | 5.43±0.22 | 5.38±0.40 | 0.97 |
| O2 | 8.12±0.45 | 8.02±0.35 | 1.05 | ND | 0.27±0.18 | |

| H1 | 7.25±0.17 | 7.07±0.20 | 1.02 | ND | 0.11±0.07 | |
|---|---|---|---|---|---|---|
| 01 | 9.78±0.12 | 8.98±0.29 | 1.19 | ND | 0.15±0.02 | |
| I | 8.21±0.45 | 9.20±0.50 | 0.87 | 4.51±0.12 | 4.23±0.26 | 1.03 |
| K1 | 8.54±0.17 | 9.43±0.46 | 0.85 | 6.12±0.15 | 5.81±0.14 | 1.05 |
| K2 | ND | 4.58±0.02 | | 3.32±0.45 | 3.45±0.53 | 0.94 |
| G3 | 9.17±0.22 | 8.71±0.22 | 1.07 | 9.54±0.17 | 9.86±0.33 | 0.95 |
| Q | 7.25±0.12 | 6.86±0.22 | 1.07 | ND | 0.17±0.14 | |
| H2 | 10.07±0.13 | 9.86±0.14 | 1.02 | 8.21±0.15 | 8.92±0.29 | 0.90 |
| L2 | 7.57±0.16 | 7.21±0.22 | 1.04 | 6.12±0.24 | 6.54±0.42 | 0.91 |
| T1 | 4.32±0.17 | 4.21±0.21 | 1.01 | ND | 0.69±0.07 | |
| M1 | 5.45±0.20 | 5.02±0.07 | 1.1 | ND | 0.17±0.04 | |
| P1 | ND | 7.17±0.28 | | 7.12±0.17 | 7.39±0.39 | 0.93 |
| P2 | 7.07±0.20 | 7.97±0.35 | 0.87 | 10.12±0.07 | 9.21±0.20 | 1.08 |
| S2 | 6.38±0.17 | 5.33±0.45 | 1.13 | ND | 0.19±0.06 | |
| S3 | 5.35±0.07 | 5.07±0.05 | 1.05 | 4.54±0.11 | 4.61±0.24 | 0.95 |
| S1 | 7.73±0.17 | 7.07±0.65 | 1.05 | ND | 0.21±0.08 | |
| R1 | ND | 2.35±0.15 | | 4.24±0.07 | 4.54±0.27 | 0.89 |
| U1 | 7.51±0.20 | 6.68±0.32 | 1.1 | ND | 0.20±0.02 | |
| W | 6.47±0.22 | 6.40±0.13 | 1.02 | ND | 0.44±0.12 | |

Table 5: simillarity between the phenotypic (P) and genotypic (G) methods by the efflux resistance in *Pseudomonas aeruginosa*

| Efflux transporter | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MexAB-OprM | | MexCD-OprJ | | MexEF-OprN | | MexXY-OprM | |
| strain | P | G | P | G | P | G | P | G |
| Reference strains | | | | | | | | |
| PAO1 | - | - | - | - | - | - | - | - |
| MutGR1 | - | - | - | - | - | - | + | + |
| JFL10 | - | - | - | - | - | - | + | + |
| PT629 | + | + | - | - | - | - | - | - |
| JFL30 | + | + | - | - | - | - | - | - |
| PAO7H | - | - | - | - | + | + | - | - |
| JFL05 | - | - | - | - | + | + | - | - |
| EryR | - | - | + | + | - | - | - | - |
| JFL28 | - | - | + | + | - | - | - | - |
| Clinical isolates | | | | | | | | |
| A1 | + | + | + | + | + | + | - | - |
| A2 | + | + | - | - | - | - | + | + |
| B | - | (#) | - | - | + | + | + | + |
| C1 | - | - | + | - | + | + | - | - |
| C2 | + | + | - | - | (*) | + | - | - |
| G4 | + | + | - | - | - | - | + | + |
| E2 | + | + | + | + | + | + | - | - |
| D1 | (*) | + | - | - | - | - | (*) | + |
| D2 | + | + | - | - | (*) | + | - | - |
| D3 | - | (#) | - | - | + | + | + | + |
| G1 | + | + | + | + | + | + | - | - |
| L1 | + | + | (*) | - | - | - | - | - |
| I | + | + | - | - | - | - | + | + |
| J | - | - | + | - | + | + | - | - |
| H1 | + | + | + | + | + | + | - | - |
| N | + | + | (*) | - | - | - | - | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| E1 | (*) | + | - | - | - | - | (*) | + |
| F | - | (#) | - | - | + | + | + | + |
| G3 | (*) | + | - | - | - | - | (*) | + |
| M1 | + | + | + | + | + | + | - | - |
| M2 | - | - | + | - | + | + | - | - |
| H2 | (*) | + | - | - | - | - | (*) | + |
| O1 | + | + | - | - | (*) | + | - | - |
| O2 | + | + | (*) | - | - | - | - | - |
| Q | + | + | - | - | (*) | + | - | - |
| K1 | + | + | - | - | - | - | + | + |
| K2 | - | (#) | - | - | + | + | + | + |
| P2 | + | + | - | - | - | - | + | + |
| L2 | (*) | + | - | - | - | - | (*) | + |
| S3 | (*) | + | - | - | - | - | (*) | + |
| S1 | + | + | + | + | + | + | - | - |
| S2 | + | + | - | - | (*) | + | - | - |
| T2 | - | - | + | - | + | + | - | - |
| P1 | - | (#) | - | - | + | + | + | + |
| T1 | + | + | (*) | - | - | - | - | - |
| U1 | + | + | - | - | (*) | + | - | - |
| P3 | - | - | + | - | + | + | - | - |
| R1 | - | (#) | - | - | + | + | + | + |
| R2 | - | - | + | - | + | + | - | - |
| W | + | + | (*) | - | - | - | - | - |

*mexA* used as the internal control in the other strains**;** (+): the efflux is most likely the cause of the promotion of the MIC (*): efflux is engaged in the elevation of the MIC : (–): efflux is unlikely to be the cause of the promotion of the MIC or there is not increase of the MIC. (#) : shows no over expression in (*mexA, mexX*) or absence in (*mexC, mexE*). Double-line boxed symbols shows full simillarity in samples; single-line boxed symbols shows parochial simillarity, and Doublezig-zag line boxed symbols shows different results.

Table1: clinical isolation of *Pseudomonas aeruginosa* collected at Alborz University-affiliated hospitals

| Patient | Gender | Sector sampling | Area of sampling |
|---|---|---|---|
| D1 | M | Infectious | Urine |
| E1 | F | PICU | Urine |
| G3 | M | Surgery | Conjunctivitis |
| H2 | M | Infectious | Urine |
| L2 | F | Surgery | Exudates |
| S3 | M | PICU | CSF |
| A2 | M | Neurology | Exudates |
| G4 | M | Emergency | Urine |
| I | M | Surgery | Conjunctivitis |
| K1 | F | Urology | Urine |
| P2 | F | Infectious | Urine |
| A1 | M | Nephrology | Exudates |
| E2 | M | Urology | Conjunctivitis |
| G1 | M | NICU | Urine |
| H1 | F | Neurology | Exudates |
| R1 | F | PICU | Urine |
| P3 | M | Surgery | Lung secretions |
| M1 | M | Emergency | Blood |
| S1 | F | Neurology | Urine |
| C2 | M | PICU | Tracheal |
| D2 | M | Nephrology | Urine |
| O1 | F | NICU | Exudates |
| Q | F | Surgery | Conjunctivitis |
| S2 | M | Urology | Conjunctivitis |
| U1 | F | Emergency | Urine |
| L1 | M | Urology | Urine |
| N | M | Neurology | Exudates |
| O2 | M | PICU | Urine |
| T1 | M | NICU | Exudates |
| W | F | Surgery | Lung secretions |
| B | M | Nephrology | Urine |

| C1 | F | Infectious | Urine |
|---|---|---|---|
| D3 | M | Nephrology | Exudates |
| J | M | NICU | Urine |
| F | F | Neurology | Urine |
| M2 | M | Infectious | Urine |
| K2 | F | Urology | Urine |
| T2 | M | PICU | Tracheal |
| P1 | M | Urology | Conjunctivitis |
| R2 | F | Nephrology | Urine |

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