Molecular Study of Efflux Genes in *Pseudomonas aeruginosa* Isolated from Clinical Samples

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**A B S T R A C T**

*Pseudomonas aeruginosa* is a common nosocomial pathogen associated with several clinical infections. It shows high resistance to most antibiotics classes due to multiple antibiotic resistance mechanisms. The aim of the present study was to assess the presence of efflux system genes by multiplex PCR among clinical isolates of *P. aeruginosa*. The study was conducted on clinical isolates of *P. aeruginosa* collected from Mansoura University hospital from January 2016 to December 2016. Identification of *P. aeruginosa* was performed according to standard microbiological techniques and antibiotics susceptibility was determined by disc diffusion methods. Multiplex PCR was used for amplification of *MexA*, *MexB*, and *MexR* genes. For detection of *OprM* and *OprD* genes, two separate rounds of PCR were performed. Isolated *P. aeruginosa* showed high resistance to gentamicin (90.6%), amikacin (66.03%) ceftazidime (77.4%), Aztreonam (64.2%), imipenem (47.2%) and ciprofloxacin (26.4%).PCR study for efflux genes revealed the presence of genes in 66.03% of *P. aeruginosa* isolates; combined *MexB* and *OprD* in 60.4%, *MexB* alone, and combined *MexB* and *OprD* in 1.9% for each. The presence of efflux genes was associated significantly with high resistance to gentamicin, ceftazidime, Cefepime, Aztreonam, Ciprofloxacin, Amikacin, Meropenem and Imipenem (P value 0.0001, 0.003, 0.0001, 0.008, 0.02, 0.03, 0.0001 and 0.0001; respectively). The present study highlights the importance of genes controlling efflux pumps as an important cause associated with *P. aeruginosa* resistance to antibiotics. Longitudinal large scales studies are required for further analysis of these genes and its expression effects on antibiotics resistance of *P. aeruginosa*.

**Introduction**

Infection with *Pseudomonas aeruginosa* (*P. aeruginosa*) is common in hospital acquired infections. It can cause a wide spectrum of infections, particularly in immune compromised patients (Aksamit, 1993).

*P. aeruginosa* has various mechanisms of resistance to antibiotics, such as broad spectrum ß-lactamases and metallo-ß-lactamases (MBL), through the alteration of penicillin-binding proteins (PBP), porin mutations, plasmid enzymatic modification, DNA-gyrase mutations, and active efflux pumps (Garza-Ramos *et al.*, 2009)

The efflux mechanisms are controlled by Resistance-Nodulation-Cell Division (RND) family operon. These RND operons are
encoded by the bacterial chromosomes. *P. aeruginosa* genome contains at least 12 structural genes for the RND efflux systems, of which four are clinically important: *MexAB-OprM* (β-lactams), *MexCD-oprJ* and *MexEF-oprN* (carbapenems and quinolones) and *mexXY-oprM* (aminoglycosides) (Poole, 2001).

However, different types of efflux pumps may extrude not only antibiotics within the same class but also different classes of antibiotics (Lister, et al., 2009).

These genes are expressed at relatively low levels, under the control of regulatory genes. Mutations in these regulators can lead to raised expression and antibiotic resistance (Sevillano et al., 2006).

Location of efflux resistance is necessary for the selection of antibiotics and its doses for treatment of infections caused by *P. aeruginosa* and for monitoring of antibiotics resistance mechanisms (Morita et al., 2012).

There are both phenotypic methods and genotypic methods for detection of efflux system. However, phenotypic methods are inaccurate with variable results (Mesaros et al., 2007). Molecular method basically depends upon the use of Real-Time PCR for gene expression. Although, this method require special equipped laboratory with the use of many probes and limited to study of two Mex systems (*MexAB-OprM* and *MexXY*) (Yoneda et al., 2005). Detection of multiple Mex genes utilizing multiplex PCR can be used as a screening tool for detection of efflux system in clinical microbiology laboratories (Mesaros et al., 2007).

The present study aims to study the presence of efflux system genes by multiplex PCR among clinical isolates of *P. aeruginosa*.

**Materials and Methods**

This study was conducted on clinical isolates of *P. aeruginosa* collected from Mansoura University hospital from January 2016 to December 2016. Clinical isolates were obtained from wound samples, blood culture and sputum samples.

Bacteriological cultures were performed according to the type of samples.

Identifications of Pseudomonas species were done according to colony morphology, characteristic pigment production of *P. aeruginosa*, gram staining, positive oxidase tests, and API 20 NE (Collee et al., 1996).

**Antibiotics susceptibility test**

Antibiotic susceptibility testing of *P. aeruginosa* was performed according to the Kirby-Bauer disk diffusion method. The used antibiotics discs were: amikacin (30 µg), gentamicin (10 µg), tobramycin, piperacillin (100 µg), piperacillin-tazobactam (100/10 µg) ceftazidime (30µg), cefepime (30µg), imipenem (10 µg), Meropenem (10µg), Aztreonam (30 µg), ciprofloxacin (5 µg), and Colistin (10 µg). According to the clinical and laboratory standards institute (CLSI) 2013.

*Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC27853 strains (American Type Culture Collection) were used as quality controls.

Plates were incubated at 37 °C for 16-18 hrs. The diameters of inhibitory zones were measured and the results were reported based on the recommendation of (CLSI) 2013.

Multidrug resistant (MDR) isolates were selected according to their non-susceptibility to at least one agent in three or more antimicrobial categories.
Purified colonies of isolated *P. aeruginosa* were subculture done Brain Heart Infusion (BHI) broth containing glycerol and kept frozen at -70ºC for molecular study.

**Bacterial DNA extraction**

Three to five colonies were used for DNA extraction by QIAamp DNA Mini Kit (Qiagen, USA) according to the manufacturer’s instructions.

**PCR amplification of efflux genes**

Multiplex PCR was used for amplification of MexA, MexB, MexR. PCR for genes OprM and OprD were performed as two separate rounds of PCR using rPSL gene was used as housekeeping control gene.

The method used for amplification and the primers sequences described previously by Arabestani et al., 2015. Primer sequences used in this study are listed in table 1.

The amplified products were subjected to gel electrophoresis by the use of 1.5% agarose gel for one hour. 100-bp DNA size marker (Qiagen, USA) was used and the agarose gel was visualized by using UV transilluminator.

**Statistical analysis**

Descriptive data were presented as frequencies and percentages via SPSS software version 18. Chi-square test was used to determine any significant difference. *P* value less than 0.05 was considered statistically significant.

**Results and Discussion**

The study included 106 *P. aeruginosa* isolates. They were isolated from wounds swabs (66%), followed by sputum (18.9%) and blood culture (15.7%). Isolated *P. aeruginosa* showed high resistance to gentamicin (90.6%), amikacin (66.03%) ceftazidime (77.4%), aztreonam (64.2%), imipenem (47.2%) and ciprofloxacin (26.4%), table 2.

PCR study for efflux genes revealed the presence of genes in 66.03% of *P. aeruginosa* isolates (Figure 1).

PCR study for efflux genes revealed the presence of genes in 66.03% of *P. aeruginosa* isolates. Mex-B, OprD in 60.4%, Mex-B alone and combined Mex-B, Opr-M in 1.9% for each (Figure 2).

The presence of efflux genes was associated significantly with high resistance to gentamicin, ceftazidime, Cefepime, aztreonam, ciprofloxacin, amikacin, meropenem and imipenem (*P* value 0.0001, 0.003, 0.0001, 0.008, 0.02, 0.03, 0.0001 and 0.0001; respectively) (Table 3).

Figure 3 shows positive *P. aeruginosa* strains for Mex-B by PCR.

*Pseudomonas aeruginosa* represents a common nosocomial pathogen causing various types of infections. This pathogen is known for its association with resistance to a wide spectrum of antibiotics (Poole, 2000).

In the present study, Isolated *P. Aeruginosa* showed high resistance to gentamicin (90.6%), amikacin (66.03%) ceftazidime (77.4%), aztreonam (64.2%), imipenem (47.2%) and ciprofloxacin (26.4%).

In previous studies, gentamicin resistance rate from 63% up to 93.7%, amikacin from 25% up to 93.4%, I mipenem from 14% up to 50% was reported (Shahcheraghi et al., 2003,Kianpouret al., 2010,Anil and Shahid,2013,Nikokar et al., 2013,Chaudhar et al., 2013). In this study, ceftazidime and
cefepime showed resistance rate of 77.4% and 75.4%; respectively. In previous studies, the resistance pattern of *P. aeruginosa* toward third and fourth classes cephalosporins have been noticed, especially in samples from wounds and burns (Jazani *et al.*, 2010; Satti *et al.*, 2011). Resistance to extended-spectrum cephalosporins usually is due to the chromosomally mediated type 1 (AmpC) beta-lactamase (Berrazeg *et al.*, 2015).

**Table 1** Genes and primer sequences used in the study

| Gene   | Sequence                                    | bp  |
|--------|---------------------------------------------|-----|
| Mex-A  | F: CTCGACCCTACGTC R: GGTTCACCTCGACACC      | 503 |
| Mex-B  | F: TACGAAGTTTCATTGAG R: AAGGTGAC GGTGATGGT | 280 |
| Mex-R  | F: GAACTACCCGTGAATC R: CACTTGGTGAGAGATGC   | 411 |
| OprM   | F: GATCCCCGACTACCAGCGCCCGG R: ATGCCGTAATGC | 247 |
| OprD   | F: ATCTACCGACAAACGTGAG R: GCCGAGGGCGATATCAAGC | 156 |
| rPSL   | F: CGTGTGACTCGAAGTA R: CGCTGTGCTCTTGTC    | 201 |

**Table 2** Antibiotics resistance among *P. aeruginosa* isolates

| Antibiotics      | Resistance |
|------------------|------------|
|                  | No | %   |
| Amikacin         | 70 | 66.03|
| Gentamicin       | 96 | 90.6 |
| Tobramycin       | 90 | 84.9 |
| Piperacillin      | 98 | 92.5 |
| Piperacillin-tazobactam | 88 | 83.0 |
| Ceftazidime      | 82 | 77.4 |
| Cefepime         | 80 | 75.5 |
| Imipenem         | 50 | 47.2 |
| Meropenem        | 55 | 51.9 |
| Aztreonam        | 68 | 64.2 |
| Ciprofloxacin    | 28 | 26.4 |
| Colistin         | 3  | 2.8  |

**Table 3** Efflux genes association with *P. aeruginosa* isolates resistant to antibiotics (n=70)

| Antibiotics       | P. aeruginosa isolates with efflux genes | P   |
|-------------------|-----------------------------------------|-----|
| Amikacin          | 26 (37.1%)                              | 0.03|
| Gentamicin        | 70 (100%)                               | 0.0001|
| Tobramycin        | 60 (85.7)                               | 0.002|
| Piperacillin      | 50 (75.1%)                              | 0.15|
| Piperacillin-tazobactam | 18 (25.7%)                       | 0.6 |
| Ceftazidime       | 58 (82.9%)                              | 0.003|
| Cefepime          | 60 (80%)                                | 0.0001|
| Imipenem          | 48 (68.6%)                              | 0.008|
| Meropenem         | 48 (68.6%)                              | 0.001|
| Aztreonam         | 68 (97.1%)                              | 0.02|
| Ciprofloxacin     | 18 (25.7%)                              | P value <0.05 is considered as statistically significant
**Fig.1** Efflux genes detected in isolated *P. aeruginosa*

**Fig.2** Efflux genes detected by PCR Combined *Mex-B, OprD* in 60.4%, *Mex-B* alone and Combined *Mex-B, Opr-M* in 1.9% for each
There are several explanations for high resistance among *P. aeruginosa* strains isolated in the present study. Among the explanations, inappropriate uses of antibiotics in patients either in community acquired infections or in hospital acquired infections leading to selection of antibiotics resistant strains. Meanwhile, *P. aeruginosa* is known to produce over 100 beta-lactamases enzymes leading to marked resistance to beta-lactams antibiotics (Queenan *et al.*, 2010).

The other mechanism associated with antibiotics resistance in *P. aeruginosa* is increased expression of efflux systems *MexAB-OprM* and *MexXY-OprA*, repression or inactivation of porin *OprD* and this limit the antibiotics entrance to the bacterium cells (Omovskaya *et al.*, 2001).

PCR study for efflux genes revealed the presence of genes in 66.03% of *P. aeruginosa* isolates; combined *MexB, OprD* in 60.4%, *MexB* alone and combined *MexB* and *OprM* in 1.9% for each. The presence of efflux genes was associated significantly with high resistance to gentamicin, ceftazidime, cefepime, aztreonam, ciprofloxacin, amikacin, meropenem and imipenem.

The association of efflux genes and resistance to various antibiotics like gentamicin, Imipenem and meropenem has been described in previous studies (Wang *et al.*, 2010, Morita *et al.*, 2012).

The efflux pumps play a significant role in multiple antibiotics resistance among *P. aeruginosa* species. These structures act by increasing the MIC concentrations of bacterial species, reducing intracellular antibiotics concentrations thus leads to the appearance of resistant strains.

The therapeutic application of efflux pumps inhibitors is a hope for development of new antibacterial therapy among different bacterial species due to the significant structural homology of efflux pumps. Researches have been concentrated on *P. aeruginosa Mex* efflux pumps and their inhibitors. In previous studies, the inhibitor had lowered the MIC values of fluoroquinolones for both sensitive and resistant strains (Omovskaya *et al.*, 2001, Mahmood *et al.*, 2016).

The present study highlights the importance of genes controlling efflux pumps as an
important cause associated with *P. aeruginosa* resistance to antibiotics. Longitudinal large scale studies are required for further analysis of these genes and its expression effects on antibiotics resistance of *P. aeruginosa*.

**Conflicts of Interest**

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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