

Original Research Article

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

Maysaa El Sayed Zaki¹, Ahmed Elewa¹ and Nashwa M. Al-Kasaby^{2*}¹Clinical Pathology department Faculty of Medicine, Mansoura University, Egypt²Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Egypt

*Corresponding author

ABSTRACT

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*.

Keywords

Multiplex PCR,
Pseudomonas aeruginosa,
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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 β -lactamases and metallo- β -l

actamases (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 μ g), gentamicin (10 μ g), tobramycin, piperacillin (100 μ g), piperacillin-tazobactam (100/10 μ g) ceftazidime (30 μ g), cefepime (30 μ g), imipenem (10 μ g), Meropenem (10 μ g), Aztreonam (30 μ g), ciprofloxacin (5 μ g), and Colistin (10 μ g). 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 don Brain Heart Infusion (BHI) broth containing glycerol and kept frozen at -70°C 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
<i>Mex-A</i>	F: CTCGACCCGATCTACGTC R: GTCTTCACCTCGACACCC	503
<i>Mex-B</i>	F: TGTCGAAGTTTTTCATTGAG R: AAGGTCAC GGTGATGGT	280
<i>Mex-R</i>	F: GAACTACCCCGTGAATC R: CACTGGTCGAGGAGATGC	411
<i>OprM</i>	F: GATCCCCGACTACCAGCGCCCCG R: ATGCGGTACTGCGCCCCGGAAGGC	247
<i>OprD</i>	F: ATCTACCGCACAAACGATGAG R: GCCGAAGCCGATATAATCAAACG	156
<i>rPSL</i>	F: GCAAGCGCATGGTCGACAAGA R: CGCTGTGCTCTTGCAGGTTGTGA	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	<i>P. aeruginosa</i> isolates with efflux genes	P
Amikacin	26 (37.1%)	0.03
Gentamicin	70 (100%)	0.0001
Tobramycin	60 (85.7)	0.002
Piperacillin	40 (57.1%)	0.15
Piperacillin-tazobactam	18 (25.7%)	0.6
ceftazidime	58(82.9%)	0.003
cefepime	56 (80%)	0.0001
Imipenem	48(68.6%)	0.0001
Meropenem	48 (68.6%)	0.0001
Aztreonam	68 (97.1%)	0.008
Ciprofloxacin	18 (25.7%)	0.02

P value <0.05 is considered as statistically significant

Fig.1 Efflux genes detected in isolated *P. aeruginosa*

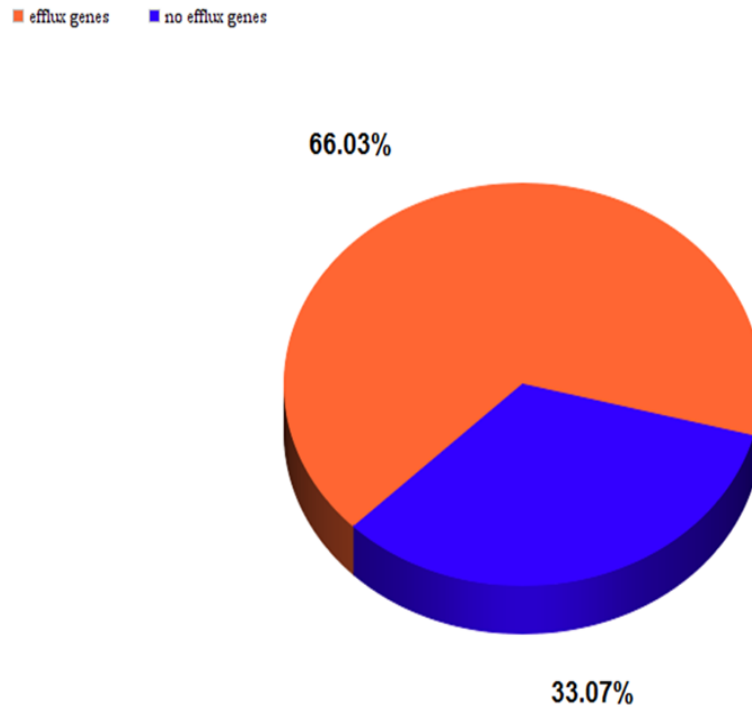


Fig.2 Efflux genes detected by PCR Combined *Mex-B, OprD* in 60.4%, *Mex-B* alone and Combined *Mex-B, Opr-M* in 1.9% for each

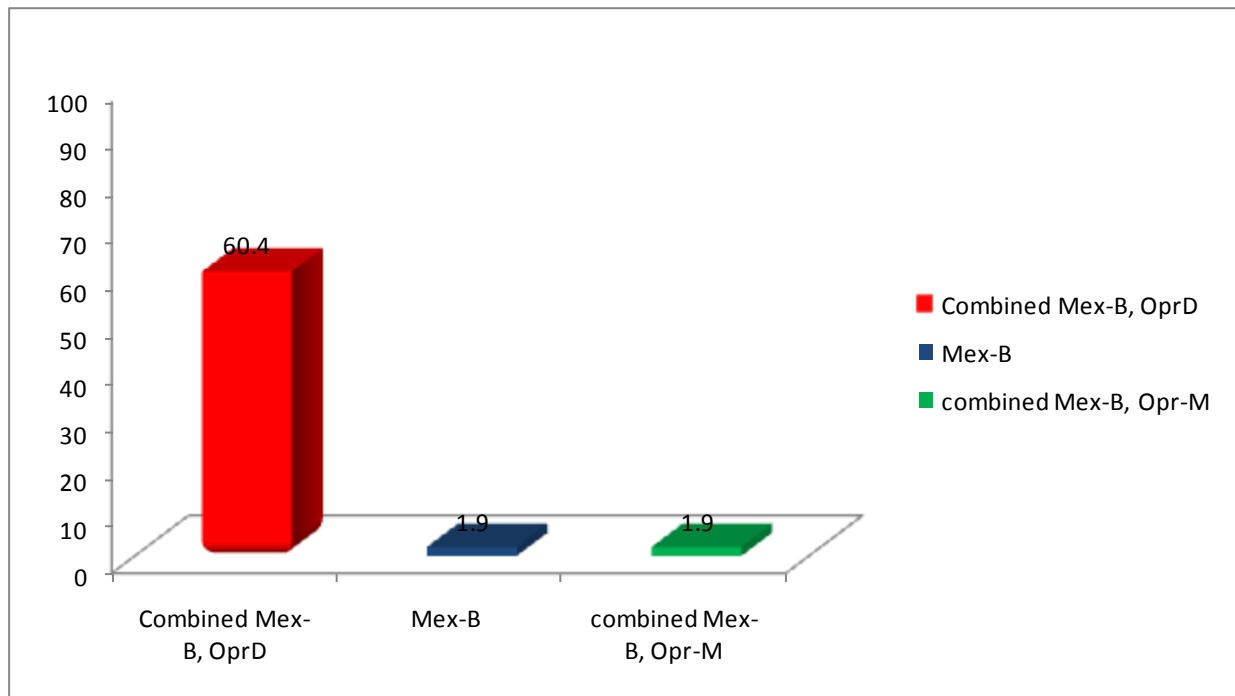


Fig.3 Positive *P. aeruginosa* strains for *Mex- B*



M: Marker; Lane1: positive isolate for *Mex- B*

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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