Molecular Characterization of Efflux Pump Mediated Resistance among Multidrug Resistant Clinical Isolates of *Pseudomonas aeruginosa*

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BACKGROUND

- *Pseudomonas aeruginosa* is an opportunistic human pathogen characterized by an innate resistance to multiple antimicrobial agents.
- A major contribution to this intrinsic multidrug resistance(MDR) is provided by a number of broadly-specific multidrug efflux systems, among which members of the Resistance Nodulation cell Division family (RND) viz., MexAB-oprM, MexXY-OprM, MexCD-OprJ & MexEF-OprN are predominant.
- MDR infections are difficult to treat with all available antibiotics and these can be controlled by combining drugs with efflux pump inhibitors (EPIs) in order to prevent treatment failure.
- Studies on efflux mediated resistance among MDR are lacking.
- Hence, this study is aimed to determine the effect of efflux pump inhibitor and the overexpression of efflux pumps in clinical isolates of *P.aeruginosa*.

Efflux pump of RND family	Substrates
MexAB-OprM	Fluoroquinolones, tetracycline, chloramphenicol and β-lactam
MexCD-OprJ	Fluoroquinolones and the antipseudomonal β-lactams (piperacillin, cefepime and meropenem)
MexXY-OprM	Fluoroquinolones, aminoglycosides, trimethoprim and chloramphenicol
MexEF-OprN	Fluoroquinolone, aminoglycoside and selected β-lactam (piperacillin, cefepime and meropenem but not carbenicillin, ceftazidime or imipenem)

MATERIALS AND METHODS

1. Strains

- ✓ A total of 213 *P. aeruginosa* clinical isolates were collected from two tertiary care hospital and these were isolated from pus(67%), tracheal wash(15%), urine(10%), blood(4%), bronchoalveolar lavage(2%), semen (1%), tracheal wash (1%).
- ✓ Identification of *P. aeruginosa* was done by standard biochemical methods.
- ✓ PAOΔ*mexR::Gm* (MexAB-OprM), PAONB(MexCD-OprJ), PAO↑EF(MexEF-OprN), PAOΔ*mexZ*::*Gm* (MexXY-OprM) were used as reference strains; PAO1 was used as control strain.

2. Antimicrobial Susceptibility Testing(AST)

- ✓ AST was performed for the following antibiotics and interpreted according to CLSI guidelines 2013.

Piperacillin (100µg)	Piperacillin/ tazobactam (100µg/10µg)	Ceftazidime (30µg)
Cefepime (30µg)	Ceftazidime/ Clavulanic acid (100µg/10µg)	Aztreonam (30µg)
Amikacin (30µg)	Gentamicin (10µg)	Netilmicin (30µg)
Tobramicin (10µg)	Ciprofloxacin (5µg)	Levofloxacin (5µg)
Ofloxacin (5µg)	Imipenem (10µg)	Meropenem (10µg)

3.Phenotypic analysis

- ❖ The following reporter antibiotics were used as phenotypic markers of the Mex efflux pumps: carbenicillin (MexAB-OprM), erythromycin (MexCD-OprJ), gentamicin (MexXY-OprM) and norfloxacin (MexEF-OprN).
- ❖ Minimum Inhibitory Concentration (MICs) was determined by microbroth dilution in the absence or presence of a broad spectrum inhibitor of Mex pumps, namely Phe-Arg β-naphthylamide (PAβN), also known as MC-207,110 at a concentration of 50µg/ml (Narcisa Mesaros *et al.*, 2007).
- ❖ Efflux was considered as possible when there was a reduction in 2log$_2$ dilution of MIC with the addition of PAβN.

4. Genotypic analysis

- ✓ Twenty one clinical isolates of *P. aeruginosa* and reference strains were harvested at the late log phase of growth and total RNA was isolated by Qiagen kit method.
- ✓ Conversion of cDNAs was carried out by Qiagen reverse transcription kit.

Detection of *mexA, mexC, mexE* and *mexX* genes by real-time PCR

SYBR green master mix was used with the following conditions: 2 min denaturation at 94ºC; 40 cycles (94ºC for 10sec, 55ºC for 1min and 72ºC for 1min); melting curve analysis 60–95ºC with continuous fluorescence readings; *rpoD* used as endogenous control.

List of primers used in this study(Quale *et al.*, 2006)

Gene	Primer Sequence 5'⟶ 3'
rpoD	F- GGGCTGTCTCGAATACGTTGA
	R- ACCTGCCGGAGGATATTTCC
mexA	F-AACCCGAACAACGAGCTG
	R- ATGGCCTTCTGCTTGACG
mexC	F-GGAAGAGCGACAGGAGGC
	R-CTGCACCGTCAGGCCCTC
mexE	TACTGGTCCTGAGCGCCT
	TCAGCGGTTGTTCGATGA
mexX	GGCTTGGTGGAAGACGTG
	GGCTGATGATCCAGTCGC

RESULTS

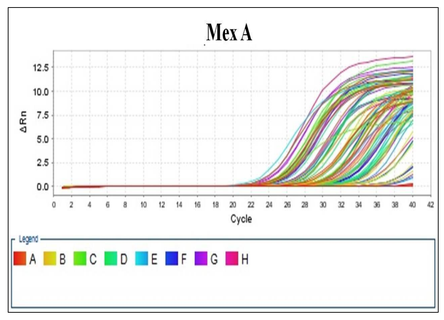
1. Phenotypic results

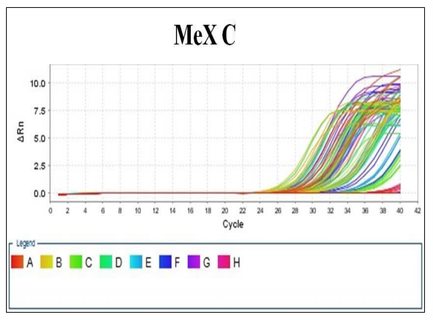
- ✓Forty isolates which were resistant to aminoglycosides, fluoroquinolones and cephalosporins by AST were chosen for the phenotypic study of efflux pump.
- ✓Efflux was considered likely if the MIC of a given strain was at least 2 log2 dilutions higher than the wild-type strain.
- ✓ For the reference strains overexpressing a mex pump, the addition of PAβN reduced the MIC values for the corresponding antibiotic markers 4 to 64 folds.
- ✓The lowest-reduction (4 fold) was observed for gentamicin MIC in PAOΔ*mexZ*::*Gm* (MexXY-OprM).
- ✓For clinical isolates, MICs difference were ranging from 2 to 32 fold for carbenicillin; 2 to 256 fold for erythromycin; 2 to 64 fold for erythromycin; 4 fold in 4, 8 in 1 and 64 in 1 strain for gentamicin.
- ✓The higher fold difference 128 and 256 were observed in 3 and 1 strain for erythromicin; 64 in 3 and 32 in 2 strains for norfloxacin; 32 in 4 strains for carbenicillin

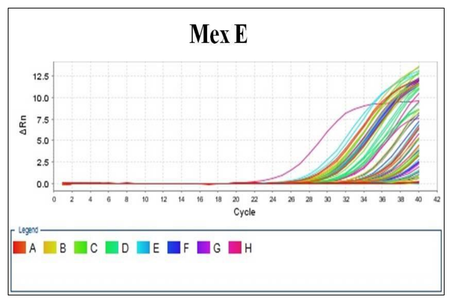
2. Genotypic results

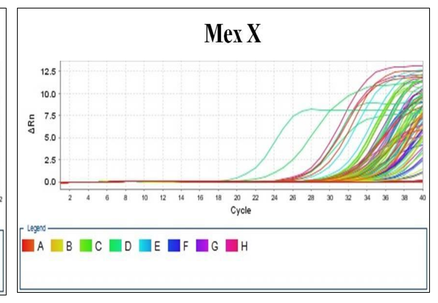
- ✓MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM efflux was considered likely for 3-fold mexA, mexC, mexE and mexX overexpression than wild type PAO1 strain by QC-RT-PCR.
- ✓mexA and mexC were overexpressed by 16 times whereas mex E and mexX were overexpressed by 6.2 and 2.14 times in reference strains.
- ✓On comparison with PAO1 strain, expression of *mexA* and *mexE* increased by 3-10 and 2-3fold respectively in all clinical isolates.
- ✓*mexC* expression ranged from 5-24 fold increase in 14(67%) isolates, 73-294 fold and 630-fold in 4 and 1 isolate respectively.
- ✓One isolate each showed 2 and 17 fold difference in *mexX* expression whereas no expression was observed for the remaining isolates.
- ✓For reference strains, a good correlation was observed between the phenotypic and genotypic methods.
- ✓For the clinical strains, the correlation was good for MexXY-OprM (18 strains with complete convergence), MexAB-OprM and MexCD-OprJ (17 strains with complete convergence) and fair for MexEF-OprN (4 strains with complete convergence).

Amplification plot

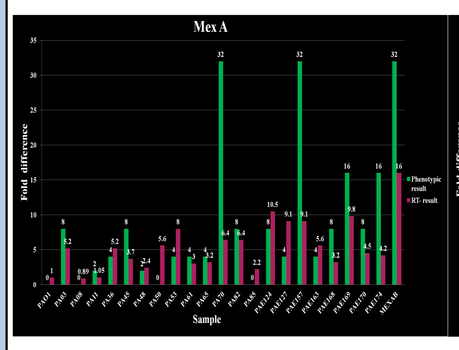


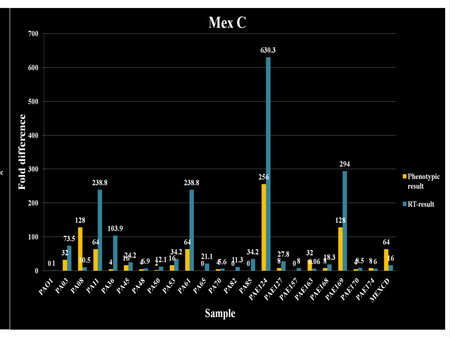


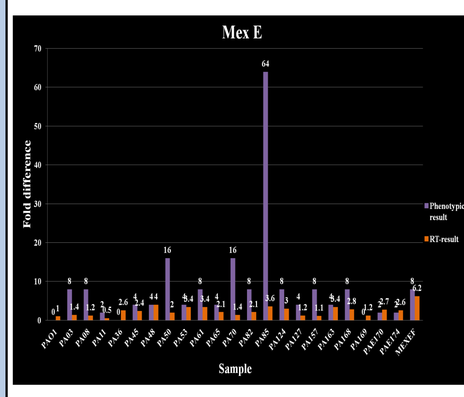


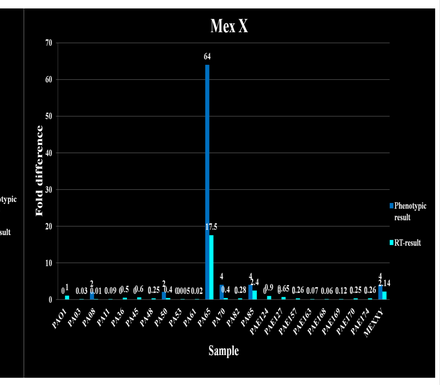


Comparison of phenotypic and genotypic results









CONCLUSION

- ✓The present study documents the prevalence of efflux pump mediated resistance among MDR isolates of *Pseudomonas aeruginosa*.
- ✓All MDR isolates over-expressed at least one of the efflux pumps.
- ✓Among the RND family genes studied, *mexC* showed a wide range as well as high levels of expression indicating its role in multidrug resistance in *P.aeruginosa*.

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