Genotypic Method is More Reliable than Phenotypic Characterization to Detect Mex Efflux Pumps in Clinical Isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Background: Active efflux significantly contributes to multiple antibiotic resistance in *P. aeruginosa*, but no simple test for its recognition in the diagnostic clinical microbiology laboratory is available today. We have developed a genotypic method allowing both a fast and accurate detection of MexAB, MexCD, MexEF, and MexXY RND transporters (ECCMID 2005, P1731). We examine here its applicability to clinical isolates and compare it with a phenotypic method (reporter antibiotics).

Methods: 16 non-duplicate clinical isolates of multi-drug resistant *P.aeruginosa* (based on resistance patterns against current antibiotics) were analyzed. MICs of selected reporter antibiotics (CAR, ERY, IMI, GEN) were determined by microdilution in MH with and without Phe-Ala-beta-Naphthylamide (PAβN; a broad spectrum inhibitor of Mex pumps). In parallel, expression of inducible *mexC* and *mexE* was evaluated by semi-quantitative RT(reverse transcription)-PCR, and that of constitutive *mexA*and *mexX*, by QC[quantitative-competitive]-RT-PCR.

Results:

Genotypic charact.			Phenotypic charact.			
pump	detection	nb strains	antibiotic	MIC		nb strains
				- PAβN	+ PAβN	
MexA	5-10 fold increase	11	CAR	> 128	32	8
				> 128	≥ 128	3
MexC	present	2	ERY	> 1024	< 16	2
MexE	present	7	IMI	2-16	0.5-4	7
MexX	8-9 fold increase	3	GEN	> 16	0.5	1
				> 16	8	2

Thus, genotypic characterization allowed detecting all 4 transporters, including in *mexA* or *mexX* positive strains showing limited changes in MIC towards reporter antibiotics in the presence of PAβN.

Conclusion: While phenotypic methods may prove useful for a first screening of efflux resistance mechanisms, genotypic methods appear more sensitive for detection because of the presence of concomitant mechanisms of resistance to the agents selected for phenotypic characterization.

INTRODUCTION

Pseudomonas aeruginosa is primarily a nosocomial pathogen, responsible for opportunistic infections. *P. aeruginosa* presents intrinsic as well as acquired resistance to a wide variety of antimicrobial agents, causing concern for efficient therapy. In this context, polyspecific efflux pumps play a key role in the multiresistance of *P. aeruginosa* towards both antibiotics and antiseptics.

Seven "Mex-type" efflux pumps able to transport various antimicrobial agents have been characterized in *P. aeruginosa*, among which 4 (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) have been shown to contribute to clinically-significant resistance levels (Llanes *et al.*, 2004).

Inhibitors of Mex efflux pumps have been described, among which Phenyl-Alanine-β-Naphthylamide (PAβN) restores antibiotic activity (at least *in vitro*) in strains resistant by overexpression of efflux pumps (Lomovskaya *et al.*, 2001).

In this context, the development of rapid and reliable methods for the early detection of this efflux pumps in clinical isolates may positively assist for the selection of appropriate therapeutic agents in a given patient and also for the screening of resistance mechanisms in epidemiological surveys.

We have recently developed a combined phenotypic and genotypic methodology for the detection of Mex-mediated resistance in *P. aeruginosa* (Mesaros *et al.*, 2005), consisting (i) in the determination of the MIC of reporter antibiotics (preferential substrates of a given Mex pump) in the absence and in the presence of PAβN, and (ii) in the evaluation of gene expression levels.

AIM OF THE STUDY

To evaluate the applicability of phenotypic and genotypic methods for the detection of resistance mediated by efflux through Mex transporters in clinical isolates of *Pseudomonas aeruginosa*.

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RESULTATS

Phenotypic characterization: MICs of *Pseudomonas aeruginosa* strains analyzed in this study. The tables show the MICs measured in the absence or in the presence of 50 mg/L Phe-Ala-β-Naphthylamide (PAβN), a broad-spectrum efflux pumps inhibitor. Antibiotics were selected for they preferential recognition by one of the Mex pumps.

Values in **green** correspond to MICs ≥ 2 dilutions than in wild type strain which are brought back to basal level with PaβN ⟶ **efflux highly probable**.

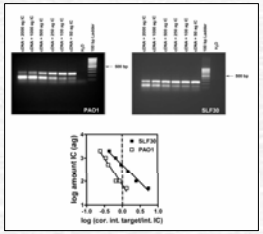
Values in **blue** correspond to MICs ≥ 2 dilutions than in wild type strain which are reduced, but not brought back to basal level with PAβN ⟶ **efflux probable**.

Values in **red** correspond to MICs ≥ 2 dilutions than in wild type strain which are marginally or not affected by PAβN OR MICs 1 dilution higher than in wild type strain ⟶ **efflux undetectable**.

Reference strains	WT (PAO1)	PT629 (AB+)	SLF30 (AB+)	EryR (CD+)	SF210 (CD+)	PAO7H (EF+)	SLF28 (EF+)	MutGR1 (XY+)	SLF05 (XY+)
Carbenicillin	16	256	256	16	32	16	16	32	32
Carbenicillin+PAβN	16	16	32(1) 16(2)	16	16	16	16	16	16
Erythromycin	128	128	128	>1024	>1024	64	128	128	128
Erythromycin+PaβN	<16	<16	<16	<16	<16	16	<16	<16	<16
Imipenem	1	1	1	1	1	4(2) 8(1)	8	1	1
Imipenem+PaβN	1	1	1	1	1	1	1	1	1
Gentamicin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	8	4
Gentamicin+PaβN	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

Clinical isolates	PA249	PA271	PA298	PA324	PA386	PA376	PA403	PA411	PA684	PA699	PA747	PA762	PA782	PA785	PA849	PA905
Carbenicillin	64	128	64	128	128	32	256	128	32	128	32	256	256	>512	>512	128
Carbenicillin+PaβN	16	16	32	32	32	16	32	32	32	32	16	256	256	128	32	32
Erythromycin	>1024	>1024	512	512	256	256	512	256	256	512	512	512	512	256	256	256
Erythromycin+PaβN	<16	<16	<16	<16	<16	<16	<16	<16	<16	64	16	16	<16	128	<16	<16
Imipenem	1	8	2	8	2	>16	2	8	0.5	2	1	8	1	1	1	1
Imipenem+PaβN	0.5	1	2	4	0.5	8	2	4	0.5	2	1	2	1	1	1	1
Gentamicin	1	0.5	0.5	1	1	1	0.5	0.5	0.5	>16	0.5	0.5	>16	>16	1	>16
Gentamicin+PaβN	0.5	0.5	0.5	1	0.25	0.25	0.5	0.5	0.5	>16	0.5	0.5	8	8	0.5	0.5

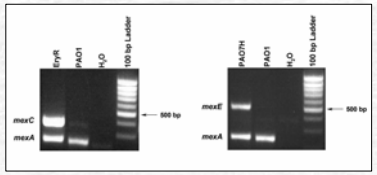
Genotypic characterization:



Quantification of *mexA*/*mexX* expression by QC-RT-PCR: *mexA* and *mexX* are constitutively expressed at a basal level. Overexpression in resistance strains can be quantified by QC-RT-PCR.

The **upper panels** illustrate the analysis of *mexA* in wild-type (PAO1) and in SLF30, a reference strain overproducing MexAB-OprM. Varying amounts of internal competitor (IC) (2000 ag to 50 ag) were co-amplified with 1 µl target cDNA for 30 cycles. Ladder in the right side is 100 bp marker.

The **lower panel** shows the quantification of *mexA* gene in wild-type (PAO1) and the MexAB-OprM overproducer SLF30. The ratio between the intensity of two bands (T and IC) was plotted in log scale as a function of known amount of IC. The amount of IC for which the intensity of two bands was equal, was taken as target cDNA concentration.



Detection of *mexE* / *mexC* expression by semi-quantitative RT-PCR: *mexE* and *mexC* are not expressed in wild-type cells. Expression in resistant strains can be detected by semi-quantitative RT-PCR. The figure illustrates RT-PCR amplification products of *mexC* and *mexE* in wild-type (PAO1) and overproducers strains (EryR – MexCD-OprJ overproducer and PAO7H – MexEF-OprN overproducer). *mexA*, which is constitutively expressed, is used here as positive control for the RT-PCR reaction.

Correlation between phenotypic and genotypic detection of resistance by efflux: 8 reference strains presenting one known efflux mechanism and 16 clinical isolates suspected of resistance by efflux have been analysed phenotypically and genotypically. The tables summarize the data obtained by both types of methodologies. In the references strains, where only one efflux pump is involved in resistance, we found a 100% correlation between phenotypic and genotypic detection. In clinical isolates, phenotypic method often gives ambiguous results (see MIC values in the table here above), as compared to what is actually detected by genotypic methods. Quantification of the expression level of constitutive genes show an 5-10-fold increase in the expression of *mexA* and *mexX* in resistant strains.

Reference strains	Phenotypic identification of Mex efflux pumps				Genotypic identification/quantification of mex efflux genes			
	MexAB-OprM	MexCD-OprJ	MexEF-OprN	MexXY-OprM	*mexA*	*mexC*	*mexE*	*mexX*
PAO1 (WT)	-	-	-	-	1	absent	absent	1
PT629 (AB+)	+	-	-	-	4.57 ± 0.11	absent	absent	~ 1
SLF30 (AB+)	+	-	-	-	6.45 ± 0.20	absent	absent	~ 1
EryR (CD+)	-	+	-	-	~ 1	present	absent	~ 1
SF210 (CD+)	-	+	-	-	~ 1	present	absent	~ 1
PAO7H (EF+)	-	-	+	-	~ 1	absent	present	~ 1
SLF28 (EF+)	-	-	+	-	~ 1	absent	present	~ 1
MutGR1 (XY+)	-	-	-	+	~ 1	absent	absent	5.58 ± 0.19
SLF05 (XY+)	-	-	-	+	~ 1	absent	absent	7.09 ± 0.17

Clinical isolates	Phenotypic identification of Mex efflux pumps				Genotypic identification/quantification of mex efflux genes			
	MexAB-OprM	MexCD-OprJ	MexEF-OprN	MexXY-OprM	*mexA*	*mexC*	*mexE*	*mexX*
PA249	+	+	-	?	5.12 ± .019	present	absent	~ 1
PA271	+	+	+	-	6.57 ± 0.15	present	present	~ 1
PA298	(+)	+	?	-	8.25 ± 0.17	absent	absent	~ 1
PA324	(+)	+	(+)	?	5.73 ± 0.16	absent	present	~ 1
PA386	(+)	?	?	?	4.79 ± 0.31	absent	present	~ 1
PA376	?	?	(+)	?	2.50 ± 0.28	absent	present	~ 1
PA403	(+)	+	?	-	6.43 ± 0.16	absent	absent	~ 1
PA411	(+)	?	(+)	-	7.71 ± 0.16	absent	present	~ 1
PA684	?	?	-	-	~ 1	absent	absent	~ 1
PA699	(+)	(+)	?	?	5.86 ± 0.07	absent	absent	~ 1
PA747	?	(+)	-	-	~ 1	absent	absent	~ 1
PA762	?	(+)	(+)	-	~ 1	absent	present	~ 1
PA782	?	+	-	(+)	~ 1	absent	absent	9.10 ± .012
PA785	(+)	?	-	(+)	7.18 ± 0.16	absent	absent	8.12 ± 0.13
PA849	(+)	?	-	?	9.86 ± 0.25	absent	absent	~ 1
PA905	(+)	?	-	+	5.96 ± 0.19	absent	absent	7.84 ± 0.17

+ : **efflux highly probable** (MICs ≥ 2 dilutions than in wild type strain which are brought back to basal level with PAβN).

(+) : efflux probable (MICs ≥ 2 dilutions than in wild type strain which are reduced, but not brought back to basal level with PAβN).

? : **efflux undetectable** (MICs ≥ 2 dilutions than in wild type strain which are marginally or not affected by PAβN or MICs 1 dilution higher than in wild type strain).

- : **efflux improbable**

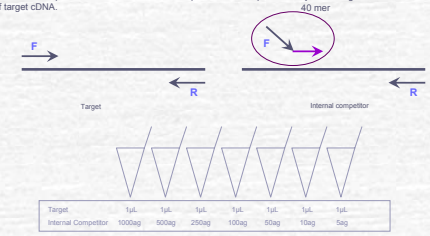
METHODS

Strains and growth conditions: we used *P. aeruginosa* wild type (PAO1), *P. aeruginosa* overexpressing MexAB-OprM (*nalB*-Kohler *et al.*, 1997 and *SLF30*-gift from J.L. Martinez, Spain), *P. aeruginosa* overexpressing MexCD-OprJ (*EryR*-M. Michea-Hamzehpour *et al.*, 1995 and *SF210*-gift from J.L. Martinez, Spain)), *P. aeruginosa* overexpressing MexEF-OprN (*PAO7H*-Kohler *et al.*, 1997 and *SLF28*-gift from J.L. Martinez, Spain)), *P. aeruginosa* overproducing MexXY-OprM (*MutGR1*-Hocquet *et al.*, 2003 and *SLF05*-gift from J.L. Martinez, Spain) and 16 clinical isolates. The strains were grown over-night on Muller-Hinton broth (MHB) at 37°C under aerobic conditions and gentle agitation (100 rpm).

MICs: Minimal inhibitory concentrations (MIC's) were determined by the broth microdilution method according to NCCLS guidelines. Inocula of 5x10^5 bacteria/ml in exponential growth were used. Measurements were done in the absence and in the presence of 50 mg/ml of the pump inhibitor Phe-Ala-β-Naphthylamide (PAβN). Reporter antibiotics that were selected as preferential substrates of a given pump were: carbenicillin for MexAB-OprM, erythromycin for MexCD-OprJ, imipenem for MexEF-OprN, and gentamicin for MexXY-OprM (Mesaros *et al.*, 2005).

RNA extraction: Total bacterial RNA (tRNA) was isolated for each strain from 1.5 ml of late-log-phase *P. aeruginosa* cultures and treated with Rnase-free DNase (1U of enzyme/µg RNA for 60 min at room temperature). All samples were checked for DNA contamination. Five µg of tRNA was used in RT-PCR reactions.

Quantification of mexA and mexX expression level by QC-RT-PCR: The genes of interest were amplified from their respective cDNA with primers -F and -R. Internal competitors were generated by PCR for each gene in the presence of primer 40mer (F+internal 20 pb) and -R primer (see below). During the PCR reactions, decreasing amounts of internal competitors (1000 ag to 5 ag) were co-amplified with the same amount of target cDNA (1 µl) (see below). PCR reactions were performed in triplicates. The two amplified products were separated in 1.7% agarose, stained with ethidium bromide, and quantified by densitometry. The concentration of internal competitor DNA at which the two amplimers show equal intensity was assigned as the concentration of target cDNA.



Detection of mexC and mexE expression by semi-quantitative RT-PCR: RNA was prepared as described for *mexA* and *mexX* and the PCR products were separeted and stained as described for *mexA* and *mexX*. To check the integrity of cDNA's, we have amplified also the *mexA* gene (that is a constitutive gene) in the same PCR.

CONCLUSIONS

- **The methods developed for the phenotypic and genotypic detection of Mex-mediated resistance are applicable to clinical isolates.**
- **Clinical isolates for which MexCD-OprM efflux is suspected on a phenotypic basis prove often negative for this pump by genotypic method, which may suggest the expression of another PAβN-sensitive pump.**
- **In reference strains overexpressing a single efflux pump, and presenting no other mechanism of resistance, phenotypic characterization gives easy-to-interpret results (with the MIC value of the reporter antibiotic returning to that observed in the wild type upon addition of PAβN). These are confirmed in all cases by genotypic methods.**
- **In clinical isolates, phenotypic characterization often leads to ambiguous conclusions, with MIC values remaining high in the presence of PAβN, probably due to the concomitant expression of other resistance mechanisms affecting the reporter antibiotics. Genotypic methods appear therefore critical for the reliable detection of resistance by efflux in *Pseudomonas aeruginosa*.**

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