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A combined phenotypic and genotypic method for the detection of Mex efflux pumps in *Pseudomonas aeruginosa*

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***Background*: Mex efflux pumps contribute to multidrug resistance in *Pseudomonas aeruginosa*. Evidencing their expression in clinical isolates would help in rationalizing antibiotic selection.**

***Methods*: We have developed a combined phenotypic and genotypic approach for the differential diagnosis of resistance mediated by four major transporters (MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM). The methodology was validated using reference strains harbouring only one specific transporter and its applicability evaluated towards seven selected clinical isolates, the resistance mechanisms of which could not be assigned by conventional techniques. Phenotypic detection used MIC measurements with reporter antibiotics [carbenicillin (MexAB-OprM); erythromycin (MexCD-OprJ); norfloxacin and imipenem (MexEF-OprN); gentamicin (MexXY-OprM)] with and without Phe-Arg-β-naphthylamide. Genotypic detection was made by semi-quantitative reverse transcription PCR (RT-PCR) for *mexC* and *mexE*, and by quantitative competitive RT-PCR and real-time PCR for *mexA* and *mexX* (correlation between both methods: >88%; overexpression levels ranging between 4.8 and 8.1).**

***Results*: Convergence between phenotypic and genotypic methods was observed in control strains for all pumps. For clinical isolates, convergence was obtained in 6 of 7 strains for MexXY-OprM and MexEF-OprM, and in 5 of 7 for MexAB-OprM and MexCD-OprJ, mostly due to hard to interpret phenotypic data.**

***Conclusions*: The data plead for combining phenotypic and genotypic approaches in the diagnosis of efflux-mediated resistance in *P. aeruginosa*.**

Keywords: multidrug resistance, Phe-Arg-β-naphthylamide, diagnostic, efflux pump, *Pseudomonas aeruginosa*

Introduction

The emergence of multidrug resistance phenotypes[1] makes epidemiological surveillance of *Pseudomonas aeruginosa* susceptibility more and more essential for the selection of successful empirical regimens. In this respect, resistance by efflux is of particular interest. Even though efflux usually confers a moderate level of resistance, its impact in the clinics could be important, because it may make antibiotics inefficient in infected sites where antibiotic concentrations are less than optimal,[2] and it confers cross-resistance to unrelated antibiotic classes in Gram-negative bacteria,[3,4] which may be difficult to anticipate based on standard, routine susceptibility testing. The Mex efflux pumps of *P. aeruginosa* are of particular interest in this context because of their exceptionally broad substrate specificity. While 12 potential efflux systems of this family have been identified in the *P. aeruginosa* genome,[5] 4 of them (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM) are best characterized as antibiotic transporters.[6]

Diagnosis of efflux-mediated resistance based only on phenotypic methods is often ill-informed due (i) to the frequent concomitant presence of other mechanisms of resistance affecting partly or totally the same drugs, (ii) to the variable expression of resistance among different strains, and (iii) to the difficulty of

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detecting mechanisms conferring low to moderate levels of resistance.[2,7] The specific detection of efflux pumps has long relied on western blot[8–11] or northern blot analysis,[12] but these methods cannot easily be implemented in the clinical laboratory. As recently presented in a review paper,[13] nucleic acid-based diagnostics are gradually replacing or complementing culture-based biochemical and immunological assays in routine microbiology laboratories, because of their speed and potentially high level of specificity. They can now be used not only for detection and identification of microbial pathogens, but also for genotyping as applied to the determination of antibiotic resistance or to microbial fingerprinting. Accordingly, semi-quantitative reverse-transcription PCR (RT-PCR),[14–17] or quantitative real-time PCR,[18–21] which directly identify the presence or the expression level of genes of interest, have been successfully used for detecting the expression of efflux pumps in *P. aeruginosa.* However, they do not provide direct information on the level of production and on the functionality of the corresponding proteins. We, therefore, have developed a combined phenotypic and genotypic approach for the detection of resistance by Mex-mediated efflux in *P. aeruginosa*, concentrating on MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM. Validation was sought with references strains, and potential applicability in the clinical setting assessed by testing a limited number of clinical isolates.

Materials and methods

Strains and growth conditions

All strains are described in Table 1. Reference strains were chosen as carrying only one specific efflux mechanism; clinical isolates were selected as 'resistance mechanism(s) difficult to assign' based on conventional interpretative analysis of resistance phenotypes by disc diffusion method. All bacteria were grown overnight in Muller–Hinton broth (Becton Dickinson & Co., Franklin Lakes, NJ, USA) at 37°C, with constant shaking at 100 rpm and under aerobic conditions.

Phenotypic analysis

The following reporter antibiotics were used as phenotypic markers of the Mex efflux pumps of interest: carbenicillin (MexAB-OprM), erythromycin (MexCD-OprJ), gentamicin (MexXY-OprM) and norfloxacin (MexEF-OprN).[3,22–25] We also tested imipenem as an indirect indicator of MexEF-OprN since it induces the expression of this pump,[26] and because its MIC is increased in MexEF-OprN overproducers (probably through a decreased expression of OprD).[10,27] MICs were determined by broth microdilution[28] 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[29] at a concentration of 50 mg/L, which fully restored the susceptibility of reference strains to the reporter antibiotics but did not impair the bacterial growth. (Lower concentrations were shown to be insufficient to reverse resistance to all substrates of a given pump;[29] large concentrations have also been used by others for clinical isolates able to express several efflux mechanisms.[30,31]) Routine assays were made with $\log_2$ dilutions. To detect minimal changes, however, arithmetic dilutions were occasionally used for carbenicillin (4 mg/L increments in the 8–32 mg/L range) and gentamicin (0.1 mg/L increments in the 0.25–1 mg/L range).

Genotypic analysis

Extraction of RNA and synthesis of cDNA

Pseudomonas aeruginosa cultures were harvested at the late log-phase of growth, based on the following considerations: (i) the expression of MexAB-OprM is phase-regulated, being minimally expressed in lag phase and increasing in log to late-log phase,[32] suggesting that collecting material at this stage of growth may improve the sensitivity of the assay; (ii) this metabolic state is reached by overnight growth, which may facilitate the routine implementation of the procedure as well as the correlation with MIC data of reporter antibiotics, which are also measured on bacterial cultures having grown overnight. Total RNA was isolated and purified as described previously.[16] Absence of residual genomic DNA was checked by PCR control reactions using RNA as template. Generation of cDNAs was carried out by reverse transcription using 5 μg of DNA-free RNA as template (Promega Reverse Transcription System, Promega Co, Madison, WI, USA). All PCR amplifications were performed using a Bio-Rad Thermocycler model 100, with the primers pairs presented in Table 2. The specificity of all these primers was checked *in silico* for absence of potential cross-hybridization [using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)], and experimentally by performing (i) PCR with each of the primer pairs and demonstrating the presence of a single band at the expected size, and (ii) real-time PCR in the absence of cDNA template and observing the absence of amplification signal.

Detection of mexA and mexX genes by real-time PCR

We used the primers *mexA3* and *mexA4*, and *mexX3* and *mexX4*, respectively, with amplification reactions performed in the presence of Sybr Green iQ™ Supermix (Bio-Rad) using a iCycler iQ® Single-Color Real-Time-PCR Detection System (MyiQ™ real time PCR software; Bio-Rad) with the following conditions: 10 min denaturation at 95°C; 40 cycles (15 s, 95°C/1 min, 60°C); melting curve analysis 60–95°C with continuous fluorescence readings; *rpsL* used as housekeeping gene.[18,19,33] The threshold cycle of samples was always within the range of standards ($R^2 > 0.995$).

Detection of mexA and mexX genes by quantitative competitive RT-PCR (QC-RT-PCR)[34]

Standard techniques were used with the following specific conditions. Internal competitor DNAs[35] were synthesized by PCR using 0.6 μM primers (*mexA-F* and *mexA-40mer-R* or *mexX-40mer-F* and *mexX-R*), 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). The PCR conditions consisted of (i) 10 min denaturation at 95°C, (ii) 30 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C for *mexA* amplification, and 30 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C for *mexX* amplification, and (iii) a final elongation at 72°C for 10 min. For quantification of the target genes expression level, different amounts of internal competitor (see Supplementary Material) were co-amplified with a constant

Table 1. Strains of *Pseudomonas aeruginosa* used in this study and MICs of the four antibiotics selected as markers for MexAB-OprM [carbenicillin (CAR)], MexCD-OprJ [erythromycin (ERY)], MexEF-OprN [norfloxacin (NOR), imipenem (IMP)] or MexXY-OprM [gentamicin (GEN)] and tested in the absence or in the presence of the wide spectrum efflux inhibitor PAβN (50 mg/L)[a]

		MIC (mg/L)[b]									
		CAR		ERY		NOR		IMP		GEN	
Strains	Reference	− PAβN	+ PAβN	− PAβN	+ PAβN	− PAβN	+ PAβN	− PAβN	+ PAβN	− PAβN	+ PAβN
Reference strains with demonstrated resistance by efflux (genotype)											
PAO1 (wild-type)		16[c]	16[c]	128	16	0.4	0.4	1	1	0.5[c]	0.5[c]
PT629 (MexAB-OprM)	24[d]	**256**	**16**	128	16	0.8	0.4	1	1	0.5	0.5
JFL30 (MexAB-OprM)	50[d]	**256**	**32(1)/16(2)**	128	16	0.8	0.4	1	1	0.5	0.5
EryR (MexCD-OprJ)	22[d]	16	16	**4096**	**16**	0.8	0.4	1	1	0.5	0.5
JFL28 (MexCD-OprJ)	50[d]	32	16	**2048**	**16**	0.8	0.4	1	1	0.5	0.5
PAO7H (MexEF-OprN)	11[d]	16	16	64	16	**3.2**	**0.2**	**4(2)/8(1)**	**1**	0.5	0.5
JFL05 (MexEF-OprN)	50[d]	16	16	128	16	**6.4**	**0.4**	**8**	**1**	0.5	0.5
MutGR1 (MexXY-OprM)	23[d]	32	16	128	16	0.4	0.4	1	1	**8**	**0.5**
JFL10 (MexXY-OprM)	50[d]	32	16	128	16	0.4	0.4	1	1	**4**	**0.5**
Clinical isolates with suspected resistance by efflux[e]											
324		**128**	**32**	**512**	**16**	**3.2**	**0.8**	*8*	*4*	1	1
376		32	16	*256*	*16*	**1.6**	**0.4**	*16*	*8*	**1**	**0.25**
411		**128**	**32**	*256*	*16*	*25.6*	*6.4*	*8*	*4*	0.5	0.5
699		**128**	**32**	*512*	*64*	*25.6*	*25.6*	2	2	*128*	*128*
762		*256*	*256*	**512**	**16**	*12.8*	*3.2*	**8**	**2**	0.5	0.5
785		*4096*	*128*	*256*	*128*	*25.6*	*25.6 (1)/12.8 (2)*	1	1	*128*	*8*
905		**128**	**32**	*256*	*16*	0.4	0.4	1	1	**16**	**0.5**

[a]Figures in bold highlight situations in which the MIC is at least 2 $\log_2$ dilutions higher than in the wild-type strain, and the addition of Phe-Arg-β-naphthylamide (PAβN) lowers the MIC to the value measured in the in wild-type strain (±1 dilution); figures in italics correspond to situations in which MIC is increased by 1 dilution only or in which MIC remains elevated upon addition of PAβN (compared with the wild strain).

[b]Three independent determinations with same values; when differences were noted between determinations, the two values are given in the table with the number of corresponding determinations between brackets.

[c]MIC by arithmetic dilutions (±PAβN): 24/16 mg/L for CAR and 0.65/0.5 mg/L for GEN.

[d]Original description of the strain.

[e]Cliniques universitaires UCL de Mont-Godinne, Yvoir, Belgium.

Table 2. Primers used in this study

Name	Sequence	Reference[a]
m*exA-F*	5′-ACCTACGAGGCCGACTACCAGA-3′	17
mexA-R	5′-GTTGGTCACCAGGGCGCCTTC-3′	17
mexA-40mer-R	5′-GTTGGTCACCAGGGCGCCTTCTGCTTGCTCACGGCCTGG-3′	this study
mexX-F	5′-CATCAGCGAACGCGAGTACAC-3′	51
mexX-R	5′-CAATTCGCGATGCGGATTG-3′	51
mexX-40mer-F	5′-CATCAGCGAACGCGAGTACACCGCAGATCGCCTCGGCCA-3′	this study
mexC-F	5′-AGCCAGCAGGACTTCGATACC-3′	17
mexC-R	5′-ACGTCGGCGAACTGCAACCGCTG-3′	17
mexE-F	5′-GTCATCGAACAACCGCTG-3′	17
mexE-R	5′-GTCGAAGTAGGCGTAGACC-3′	17
mexA3	5′-AAGCGCCTGTTCAAGGAAGG-3′	this study
mexA4	5′-CCTTGGTGTAGCGCAGGTTG-3′	this study
mexX3	5′-CCGTGCTGTTCCAGATCGAC-3′	this study
mexX4	5′-TCCTTGATCAGGTCGGCGTA-3′	this study
rpsL-S	5′-CGGCACTGCGTAAGGTATGC-3′	this study
rpsL-AS	5′-CCCGGAAGGTCCTTTACAC-3′	this study

[a]Original description of the sequence.

amount of target cDNA. Amplifications were carried out as described for the synthesis of the internal competitors, but using the primer pairs *mexA-F* and *mexA-R* (252 bp) or *mexX-F* and *mexX-R* (451 bp). Band intensities of each product were analysed optically [Gel-Doc 1000 camera system operated with the QuantityOne® image analysis software (Bio-Rad, Richmond, CA, USA)]. Raw data were corrected for the size of the amplified fragments.[36]

Detection of mexC *and* mexE *expression by semi-quantitative RT-PCR*

We used a published method[17] with the primers listed in Table 2. For a positive control, *mexA* (expressed at a basal level in all strains)[37] was amplified in parallel.

Assessment of reproducibility and sensitivity of the quantitative methods

MICs were determined in triplicate using independent cultures (differences observed are shown in the Results) and cDNA preparation was done in triplicate from independent bacterial cultures. The PCR quantification reactions were routinely performed in triplicate for each of the cDNA preparations, generating a total of nine data sets for each strain. The intra-day ($n = 3$) and inter-day ($n = 9$) coefficients of variations (%) were 16.1 and 7.9 for *mexA*, and 10.3 and 8.3 for *mexX* in QC-RT-PCR; and 12.3 and 7.3 for *mexA*, and 9.2 and 9.6 for *mexX* in real-time PCR. The lowest limit of detection (i.e. lowest concentration of the standard giving a highly reproducible signal) was 10^2 copies/sample by real time PCR and 3.5×10^2 copies/sample by QC-RT-PCR.

Definition of convergence/divergence between the phenotypic and the genotypic methods

In the phenotypic analysis, efflux was considered likely if the MIC of a given strain was at least 2 $\log_2$ dilutions higher than in the wild-type strain, and if the addition of PAβN lowered its value to that which was measured in the wild-type strain (± 1 dilution). If the MIC was increased by 1 dilution only or if the MIC remained elevated upon addition of PAβN (compared with the wild strain), efflux was considered as possible. In the genotypic analysis: (i) MexAB-OprM- and MexXY-OprM-based efflux was considered likely for a $\geq$3-fold *mexA* and *mexX* over-expression by QC-RT-PCR or real-time PCR compared with the wild strain; (ii) MexCD-OprJ- and MexEF-OprN-based efflux was considered likely if *mexC* and *mexE* were detected. Convergence between the phenotypic and genotypic methods was considered as complete if both methods showed the likely presence (or absence) of the corresponding efflux mechanism, and partial if efflux was scored only as possible by the phenotypic approach, while being scored as likely by the genotypic approach. All other situations were scored as denoting divergence.

Materials

Carbenicillin and norfloxacin were purchased from Sigma-Aldrich Co, St Louis, MO, USA. Gentamicin, imipenem and erythromycin were obtained as the corresponding branded products distributed for clinical usage in Belgium by Schering-Plough, Merck Sharp and Dohme, and Abbott, respectively. PAβN was obtained from Sigma-Aldrich and, upon its discontinuation from their product list, received from Mpex Pharmaceuticals Inc., San Diego, CA, USA. Unless stated otherwise, all other reagents were obtained from E. Merck AG, Darmstadt, Germany, or Sigma-Aldrich.

Results

Phenotypic characterization of resistance mediated by Mex efflux pumps

Table 1 shows the MIC values measured in the absence and in the presence of 50 mg/L of the broad-spectrum efflux inhibitor

PAβN. For reference strains, there was a $\geq 2 \log_2$ dilutions increase of MICs for each reporter antibiotic according to their established resistance mechanism, and the addition of PAβN brought these values to, or close to (1 dilution), those of the wild-type strain. Addition of PAβN in PAO1 caused a 33 and 23% reduction of the MICs of carbenicillin and gentamicin, respectively (based on arithmetic dilutions measurements), denoting a low, basal expression of MexAB-OprM and MexXY-OprM.[9,38] The MIC of erythromycin was markedly reduced in PAO1 by addition of PAβN, suggesting the presence of another PAβN-inhibitable efflux transporter in this strain (erythromycin has been described so far as a potential substrate of MexJK and MexVW[39]). Considering the 7 clinical isolates, MICs 2 to 3 $\log_2$ dilutions higher than in the wild-type strain PAO1 were observed in 6 strains for carbenicillin, in 3 strains for erythromycin, in 6 strains for norfloxacin, in 4 strains for imipenem, and in 3 strains for gentamicin. The pattern of restoration of activity by PAβN led us to suspect that MexAB-OprM was active in strains 324, 411, 699, 785 and 905; MexCD-OprJ in strains 324, 699 and 762; MexEF-OprN in strains 324, 376, 411 and 762, and MexXY-OprM in strains 376, 785 and 905. Isolate 376 showed a non-significant increase of MIC compared with the wild-type strain PAO1 but PAβN decreased this value by 2 dilutions.

Genotypic characterization of resistance mediated by Mex efflux pumps

Three techniques were used for this analysis. For *mexA* and *mexX*, methods quantifying the amount of mRNA were considered essential because these genes are constitutively expressed at a basal level in wild-type strains (contributing to intrinsic resistance), and overexpressed to variable levels in resistant strains.[37,40] We therefore designed a real-time PCR assay, which was validated by a parallel examination of the samples using QC-RT-PCR assay [see Supplementary Data—available at JAC Online (http://jac.oxfordjournals.org/)]. For *mexC* and *mexE*, a semi-quantitative method was considered sufficient because the expression of these genes is strictly down-regulated in wild-type strains but expressed to high levels in resistant strains.[10,17,37,41] Thus, we selected a semi-quantitative PCR assay, which allowed us to easily detect the appearance of an amplification product in resistant strains only [see Supplementary Data—available at JAC Online (http://jac.oxfordjournals.org/)].

Determination of the overexpression of mexA and mexX

Table 3 shows that *mexA* and *mexX* were overexpressed by 4 to 7 times in reference strains. Similar overexpression levels were detected in 6 and 3 clinical isolates for *mexA* and *mexX*, respectively. A good agreement was observed between the overexpression levels detected by real-time PCR or by QC-RT-PCR.

Determination of mexC and mexE expression by semi-quantitative RT-PCR

In reference strains, *mexC* and *mexE* were specifically amplified in EryR and JFL28, and in PAO7H and JFL05, respectively, the other strains showing only the amplification of *mexA* used as the internal control. This technique was then applied to the clinical isolates included in the study, and the results (expression of the corresponding genes or not) are shown in Table 4.

Table 3. Quantification of the expression levels *of mexA* and *mexX* genes by QC-RT-PCR and real time PCR and correlation between the values determined by the two techniques

	mexA expression level			*mexX* expression level		
Strain	QC-RT-PCR	real time PCR	QC-RT-PCR/ real time PCR	QC-RT-PCR	real time PCR	QC-RT-PCR/ real time PCR
Reference strains (with demonstrated genotype of specific transporter)						
PAO1	1[a]	1[b]		1[c]	1[b]	
PT629	4.57 ± 0.11	4.18 ± 0.14	1.09	ND	0.18 ± 0.01	
JFL30	6.45 ± 0.20	6.04 ± 0.07	1.07	ND	1.18 ± 0.02	
MutGR1	ND	1.53 ± 0.17		5.58 ± 0.19	6.38 ± 0.40	0.87
JFL10	ND	1.27 ± 0.09		7.09 ± 0.17	6.61 ± 0.07	1.07
Uncharacterized clinical isolates[d]						
324[e]	5.73 ± 0.16	5.45 ± 0.45	1.05	ND	0.14 ± 0.03	
376[f]	ND	2.50 ± 0.28		4.77 ± 0.18	5.07 ± 0.87	0.94
411[e]	7.71 ± 0.16	6.90 ± 0.37	1.12	ND	0.19 ± 0.02	
699[e]	5.86 ± 0.07	5.84 ± 0.34	1.01	ND	0.67 ± 0.17	
785[e,g]	7.18 ± 0.16	6.74 ± 0.29	1.07	8.12 ± 0.13	8.63 ± 0.21	0.94
905[e,g]	5.96 ± 0.19	6.72 ± 0.43	0.87	7.84 ± 0.17	7.46 ± 0.34	1.05

ND, not determined.
[a]Basal expression level: 19520 ± 2440 copies/5 μg tRNA (QC-RT-PCR).
[b]Ratio of gene expression between the target gene (*mexA, mexX*) and the reference gene (*rpsL*).
[c]Basal expression level: 2440 ± 360 copies/5 μg of tRNA (QC-RT-PCR).
[d]Analysis performed only for strains suspected to overexpress *mexA* or *mexX* based on phenotypic data (see e-g).
[e]Suspected to harbour the MexAB-OprM-based resistance mechanism (as from data of Table 1).
[f]Low level of resistance to gentamicin with 4-fold decrease in MIC in the presence of PAβN (as from data of Table 1).
[g]Suspected to harbour the MexXY-OprM-based resistance mechanism (as from data of Table 1).

Table 4. Convergence of the phenotypic (P)[a] and genotypic (G)[b] methods for the detection of Mex-mediated efflux in reference strains and clinical isolates

	Efflux transporter							
	MexAB OprM		MexCD OprJ		MexEF OprN		MexXY OprM	
Strains	P	G	P	G	P[c]	G	P	G
Reference strains (with demonstrated genotype of specific transporter)								
PAO1	−	−	−	−	−	−	−	−
PT629	+	+	−	−	−	−	−	−
JFL30	+	+	−	−	−	−	−	−
EryR	−	−	+	+	−	−	−	−
JFL28	−	−	+	+	−	−	−	−
PAO7H	−	−	−	−	+	+	−	−
JFL05	−	−	−	−	+	+	−	−
MutGR1	−	−	−	−	−	−	+	+
JFL10	−	−	−	−	−	−	+	+
Uncharacterized clinical isolates[d]								
324	+	+	+	+	+	+	-	-
376	-	(-)	-	-	+	+	+	+
411	+	+	-	-	(+)	+	-	-
699	+	+	(+)	-	-	-	-	-
762	-	-	+	-	+	+	-	-
785	(+)	+	-	-	-	-	(+)	+
905	+	+	-	-	-	-	+	+

[a]Phenotypic method key: +, efflux is most likely the cause of the elevation of the MIC (the corresponding figures are in bold in Table 1); (+), efflux is participating in the elevation of the MIC (the corresponding figures are in italic in Table 1); −, efflux is unlikely to be the cause of the elevation of the MIC (the corresponding figures are in plain character in Table 1) or there is no elevation of the MIC.
[b]Genotypic method key: +, overexpression (*mexA*, *mexX*) or presence (*mexC*, *mexE*); (−), low level of expression *(mexA*); −, no overexpression (*mexA*, *mexX*) or absence (*mexC*, *mexE*).
[c]Interpretation based on the combined results obtained with norfloxacin and imipenem; the efflux is suspected (and a score of + is given) if either norfloxacin or imipenem MICs (or both) are returned to wild-type values in the presence of PAβN.
[d]Double-line boxed symbols, full convergence; single-line boxed symbols, partial convergence (phenotypic evidence for a role of efflux is weaker than suggested from genotypic data); zig-zag line boxed symbols, divergent results.

Applicability of phenotypic and genotypic methods to the diagnosis of Mex-mediated resistance in clinical isolates

Table 4 summarizes and compares the results of the phenotypic and the genotypic methods as applied to the reference strains and the 7 clinical isolates. For reference strains, an excellent correlation was observed between the two methods. For the clinical strains, the correlation was excellent for MexEF-OprN and MexXY-OprM (6 strains with complete convergence; 1 strain with partial convergence; no divergence), good for MexAB-OprM (5 strains with complete convergence; 2 strains with partial convergence; no divergence), and fair for MexCD-OprJ (5 strains with complete convergence; 1 strain with partial convergence; 1 strain with divergence; for the latter strain, the phenotypic method yielded a positive or likely positive result for the presence of an efflux-mediated resistance, but the semi-quantitative genotypic method was negative).

Discussion

The present study proposes a combined phenotypic and genotypic approach for the specific diagnostic of resistance mediated by four Mex efflux pumps in *P. aeruginosa*. MexAB-OprM and MexXY-OprM are expressed at a basal level in wild-type strains, contributing to the low intrinsic susceptibility of *P. aeruginosa* to the corresponding antibiotics, but are overproduced in resistant strains.[9,23,38,42] MexCD-OprJ and

MexEF-OprN are not detected in standard conditions of culture for wild-type bacteria, but their expression is up-regulated in resistant bacteria.[8,10,16] As opposed to diagnostic methods based solely on MIC determinations towards a single antibiotic,[43] or using a non-specific inhibitor such as CCCP,[15] the phenotypic approach devised here provides a first level of differentiation among these four Mex efflux pumps. However, and as already noted by others,[7] interpretation of phenotypic data remains difficult with clinical strains, probably because of the co-expression of resistance mechanisms other than efflux. While interpretation could be made easier by increasing the number of reporter antibiotics, this would considerably weigh down the interest of the method. Using more specific inhibitors[44] faces the problem of lack of commercial supply of the corresponding products.

Specific methods to directly evidence the genes encoding the proteins responsible for efflux appear, therefore, desirable. For detecting overexpression of constitutively expressed pumps, quantitative assays are necessary. We show here that two methods can be used interchangeably, namely real-time PCR (rapid but expensive) or QC-RT-PCR (laborious but less expensive). This conclusion, and the levels of overexpression observed, are in accordance with what has been demonstrated in other applications,[45,46] as well as in the present application with different amplicons and other clinical strains.[18,19,33,47] Of the two, real-time PCR technology is more likely to become the standard method in routine, based on a series of advantages which include excellent sensitivity and specificity, low contamination risk, and speed,[48] and on the development of high throughput systems, allowing for the concomitant run of several reactions in parallel. However, we did not apply this expensive method for the detection of the genes of inducible pumps (MexCD-OprJ and MexEF-OprN), since all previous studies consistently show that the expression of *mexC* or *mexE* is strictly down-regulated in wild-type strains,[10,41] but is large (100–1000× the wild-type value) in *nfxB* or *nfxC* resistant mutants.[14,18] Inclusion of the detection of *mexA*, a constitutively expressed gene, as control decreases the risk of false negatives.

Genotypic diagnostic methods do not necessarily provide information on the final expression of the gene product and its functionality.[19] We addressed this issue by examining the correlation between the genotypic and the phenotypic methods developed here (Figure 1). It clearly appears that the use of combined thresholds—namely ≥ 3 for gene overexpression (close to what was proposed in an independent recent study[21]), and a $\geq 1.5\ \log_2$ dilution reduction of MIC—allows differentiation between low, probably meaningless, changes from those that may require reconsideration of the susceptibility pattern of the corresponding clinical isolate.

Besides the technical and potential clinical interest of the diagnostic methodology presented here, some biological observations made during our study also deserve attention. First, we have shown that the basal expression level of *mexX* is much lower than that of *mexA* but that both efflux pumps are overexpressed 4 to 8 times in resistant strains, suggesting that a lower quantity of MexXY-OprM than MexAB-OprM protein may be needed for effective transport of the corresponding substrates. Second, and as in another study,[8] overexpression of *mexX* in clinical isolates is systematically associated with that of *mexA*. This may be related to the fact that MexXY uses OprM as a porin,[9] the expression of which is under the control of the regulator of the MexAB-OprM operon.[49] Third, PAβN was found to reduce the MIC of imipenem in MexEF-OprN reference strains (PAO7H and JFL05), as if it were a substrate of this pump. Potential reasons may include a partial transport of imipenem by MexEF-OprN, or by a still uncharacterized Mex pump inhibitable by PAβN, or an action of PAβN on the common regulator, causing a reduction in the pump expression and an increased expression of the OprD porin. Fourth, clinical isolates were found to express several efflux pumps at the same time, causing particularly complex multidrug-resistant phenotypes. Even though these conclusions still need to be considered with caution due to the small number of strains included in the present study, our data plead for a larger implementation of diagnosis of efflux, through the combined use of appropriate phenotypic and genotypic methods in routine clinical microbiology, for the correct identification of resistance mechanisms in these organisms. This knowledge may be helpful, at the level of the individual patient, for rationalizing the antibiotic choice and dosing and, at the level of the hospital, for defining antibiotic policies, based on epidemiological surveys evidencing the most prevailing resistance mechanisms.

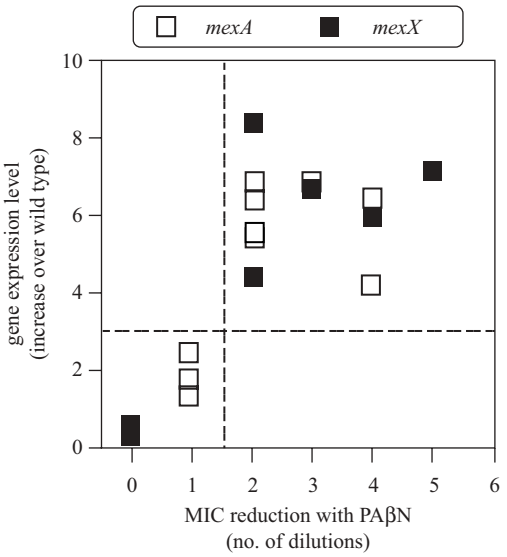

Figure 1. Correlation between the level of expression of constitutive Mex pumps and the effect of PAβN on the MICs of reporter antibiotics (carbenicillin for *mexA* and gentamicin for *mexX*). Data are expressed as the level of overexpression by comparison with the wild-type strain PAO1, as determined by real-time PCR (see Table 3) and as decrease in MIC upon addition of PAβN, expressed in number of dilutions. The dotted lines are arbitrarily set at a value of 3 for gene overexpression and at 1.5 $\log_2$ dilution for MIC reduction caused by addition of PAβN. Data are then grouped in two quadrants of potentially different diagnostic significance (lower left, no or minimally meaningful efflux-mediated decrease of susceptibility; upper right, efflux is likely to be the cause of the decreased susceptibility).

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Transparency declarations

None to declare.

Supplementary data

Supplementary data are available at JAC Online (http://jac.oxfordjournals.org/).

References

1. Rossolini GM, Mantengoli E. Treatment and control of severe infections caused by multiresistant *Pseudomonas aeruginosa*. *Clin Microbiol Infect* 2005; **11** Suppl 4: 17–32.

2. Hocquet D, Patry I, Dupont P *et al*. Low-level resistance to fluoroquinolones conferred by efflux overproduction in *Pseudomonas aeruginosa*: clinical significance and routine detection. *Pathol Biol (Paris)* 2005; **53**: 495–9.

3. Aeschlimann JR. The role of multidrug efflux pumps in the antibiotic resistance of *Pseudomonas aeruginosa* and other gram-negative bacteria. Insights from the Society of Infectious Diseases Pharmacists. *Pharmacotherapy* 2003; **23**: 916–24.

4. Poole K. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. *J Mol Microbiol Biotechnol* 2001; **3**: 255–64.

5. Stover CK, Pham XQ, Erwin AL *et al*. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. *Nature* 2000; **406**: 959–64.

6. Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria. *Drugs* 2004; **64**: 159–204.

7. Vedel G. Simple method to determine β-lactam resistance phenotypes in *Pseudomonas aeruginosa* using the disc agar diffusion test. *J Antimicrob Chemother* 2005; **56**: 657–64.

8. Llanes C, Hocquet D, Vogne C *et al*. Clinical strains of *Pseudomonas aeruginosa* overproducing MexAB-OprM and MexXY efflux pumps simultaneously. *Antimicrob Agents Chemother* 2004; **48**: 1797–802.

9. Masuda N, Sakagawa E, Ohya S *et al*. Contribution of the MexX-MexY-oprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2000; **44**: 2242–6.

10. Kohler T, Michea-Hamzehpour M, Henze U *et al*. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. *Mol Microbiol* 1997; **23**: 345–54.

11. Niga T, Ito H, Oyamada Y *et al*. Cooperation between alteration of DNA gyrase genes and over-expression of MexB and MexX confers high-level fluoroquinolone resistance in *Pseudomonas aeruginosa* strains isolated from a patient who received a liver transplant followed by treatment with fluoroquinolones. *Microbiol Immunol* 2005; **49**: 443–6.

12. De Kievit TR, Parkins MD, Gillis RJ *et al*. Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 2001; **45**: 1761–70.

13. Jannes G, De Vos D. A review of current and future molecular diagnostic tests for use in the microbiology laboratory. *Methods Mol Biol* 2006; **345**: 1–21.

14. Wolter DJ, Smith-Moland E, Goering RV *et al*. Multidrug resistance associated with *mexXY* expression in clinical isolates of *Pseudomonas aeruginosa* from a Texas hospital. *Diagn Microbiol Infect Dis* 2004; **50**: 43–50.

15. Pournaras S, Maniati M, Spanakis N *et al*. Spread of efflux pump-overexpressing, non-metallo-β-lactamase-producing, meropenem-resistant but ceftazidime-susceptible *Pseudomonas aeruginosa* in a region with blaVIM endemicity. *J Antimicrob Chemother* 2005; **56**: 761–4.

16. Morita Y, Murata T, Mima T *et al*. Induction of mexCD-oprJ operon for a multidrug efflux pump by disinfectants in wild-type *Pseudomonas aeruginosa* PAO1. *J Antimicrob Chemother* 2003; **51**: 991–4.

17. Li XZ, Barre N, Poole K. Influence of the MexA-MexB-oprM multidrug efflux system on expression of the MexC-MexD-oprJ and MexE-MexF-oprN multidrug efflux systems in *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 2000; **46**: 885–93.

18. Dumas JL, Van Delden C, Perron K *et al*. Analysis of antibiotic resistance gene expression in *Pseudomonas aeruginosa* by quantitative real-time-PCR. *FEMS Microbiol Lett* 2006; **254**: 217–25.

19. Yoneda K, Chikumi H, Murata T *et al*. Measurement of *Pseudomonas aeruginosa* multidrug efflux pumps by quantitative real-time polymerase chain reaction. *FEMS Microbiol Lett* 2005; **243**: 125–31.

20. Quale J, Bratu S, Gupta J *et al*. Interplay of efflux system, ampC, and oprD expression in carbapenem resistance of *Pseudomonas aeruginosa* clinical isolates. *Antimicrob Agents Chemother* 2006; **50**: 1633–41.

21. Hocquet D, Nordmann P, El Garch F *et al*. Involvement of the MexXY-OprM efflux system in emergence of cefepime resistance in clinical strains of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2006; **50**: 1347–51.

22. Hamzehpour MM, Pechere JC, Plesiat P *et al*. OprK and OprM define two genetically distinct multidrug efflux systems in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1995; **39**: 2392–6.

23. Hocquet D, Vogne C, El Garch F *et al*. MexXY-OprM efflux pump is necessary for adaptive resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother* 2003; **47**, 1371–5.

24. Kohler T, Michea-Hamzehpour M, Plesiat P *et al*. Differential selection of multidrug efflux systems by quinolones in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1997; **41**: 2540–3.

25. Maseda H, Saito K, Nakajima A *et al*. Variation of the *mexT* gene, a regulator of the MexEF-oprN efflux pump expression in wild-type strains of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 2000; **192**: 107–12.

26. Kolayli F, Karadenizli A, Savli H *et al*. Effect of carbapenems on the transcriptional expression of the *oprD*, *oprM* and *oprN* genes in *Pseudomonas aeruginosa*. *J Med Microbiol* 2004; **53**: 915–20.

27. Kohler T, Epp SF, Curty LK *et al*. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. *J Bacteriol* 1999; **181**: 6300–5.

28. Anonymous. *Performance Standards for Antimicrobial Susceptibility Testing (16th Informational Supplement)—document M100-S16*. Clinical and Laboratory Standards Institute, Wayne, Pa, USA, 2006.

29. Lomovskaya O, Warren MS, Lee A *et al*. Identification and characterization of inhibitors of multidrug resistance efflux pumps in *Pseudomonas aeruginosa*: novel agents for combination therapy. *Antimicrob Agents Chemother* 2001; **45**: 105–16.

30. Coban AY, Ekinci B, Durupinar B. A multidrug efflux pump inhibitor reduces fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates. *Chemotherapy* 2004; **50**: 22–6.

31. Kern WV, Steinke P, Schumacher A *et al*. Effect of 1-(1-naphthylmethyl)-piperazine, a novel putative efflux pump inhibitor, on antimicrobial drug susceptibility in clinical isolates of *Escherichia coli*. *J Antimicrob Chemother* 2006; **57**: 339–43.

32. Evans K, Poole K. The MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa* is growth-phase regulated. *FEMS Microbiol Lett* 1999; **173**: 35–9.

33. El Amin N, Giske CG, Jalal S *et al*. Carbapenem resistance mechanisms in *Pseudomonas aeruginosa*: alterations of porin OprD and efflux proteins do not fully explain resistance patterns observed in clinical isolates. *APMIS* 2005; **113**: 187–96.

34. Gilliland G, Perrin S, Blanchard K *et al*. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. *Proc Natl Acad Sci USA* 1990; **87**: 2725–9.

35. Celi FS, Zenilman ME, Shuldiner AR. A rapid and versatile method to synthesize internal standards for competitive PCR. *Nucleic Acids Res.* 1993; **21**: 1047.

36. Skarits C, Fischer S, Haas OA. Quantitation of the sterol regulatory element-binding protein mRNA in mononuclear blood cells by competitive RT-PCR. *Clin Chim Acta* 2003; **336**: 27–37.

37. Poole K, Srikumar R. Multidrug efflux in *Pseudomonas aeruginosa*: components, mechanisms and clinical significance. *Curr Top Med Chem* 2001; **1**: 59–71.

38. Li XZ, Nikaido H, Poole K. Role of mexA-mexB-oprM in antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1995; **39**: 1948–53.

39. Poole K. Efflux-mediated multiresistance in Gram-negative bacteria. *Clin Microbiol Infect* 2004; **10**: 12–26.

40. Jo JT, Brinkman FS, Hancock RE. Aminoglycoside efflux in *Pseudomonas aeruginosa*: involvement of novel outer membrane proteins. *Antimicrob Agents Chemother* 2003; **47**: 1101–11.

41. Poole K, Gotoh N, Tsujimoto H *et al*. Overexpression of the mexC-mexD-oprJ efflux operon in nfxB-type multidrug-resistant strains of *Pseudomonas aeruginosa*. *Mol Microbiol* 1996; **21**, 713–24.

42. Poole K, Krebes K, McNally C *et al*. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. *J Bacteriol* 1993; **175**: 7363–72.

43. Kriengkauykiat J, Porter E, Lomovskaya O *et al*. Use of an efflux pump inhibitor to determine the prevalence of efflux pump-mediated fluoroquinolone resistance and multidrug resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2005; **49**: 565–70.

44. Yoshida K, Nakayama K, Kuru N *et al*. MexAB-OprM specific efflux pump inhibitors in *Pseudomonas aeruginosa*. Part 5: Carbon-substituted analogues at the C-2 position. *Bioorg Med Chem* 2006; **14**: 1993–2004.

45. Achenbach JE, Topliff CL, Vassilev VB *et al*. Detection and quantitation of bovine respiratory syncytial virus using real-time quantitative RT-PCR and quantitative competitive RT-PCR assays. *J Virol Methods* 2004; **121**: 1–6.

46. Kim Y, Gharaibeh SM, Stedman NL *et al*. Comparison and verification of quantitative competitive reverse transcription polymerase chain reaction (QC-RT-PCR) and real time RT-PCR for avian leukosis virus subgroup J. *J Virol Methods* 2002; **102**: 1–8.

47. Islam S, Jalal S, Wretlind B. Expression of the MexXY efflux pump in amikacin-resistant isolates of *Pseudomonas aeruginosa*. *Clin Microbiol Infect* 2004; **10**: 877–83.

48. Espy MJ, Uhl JR, Sloan LM *et al*. Real-time PCR in clinical microbiology: applications for routine laboratory testing. *Clin Microbiol Rev* 2006; **19**: 165–256.

49. Poole K, Tetro K, Zhao Q *et al*. Expression of the multidrug resistance operon mexA-mexB-oprM in *Pseudomonas aeruginosa*: mexR encodes a regulator of operon expression. *Antimicrob Agents Chemother* 1996; **40**: 2021–8.

50. Linares JF, Lopez JA, Camafeita E *et al*. Overexpression of the multidrug efflux pumps MexCD-OprJ and MexEF-OprN is associated with a reduction of type III secretion in *Pseudomonas aeruginosa*. *J Bacteriol* 2005; **187**: 1384–91.

51. Sobel ML, McKay GA, Poole K. Contribution of the MexXY multidrug transporter to aminoglycoside resistance in *Pseudomonas aeruginosa* clinical isolates. *Antimicrob Agents Chemother* 2003; **47**: 3202–7.