Open Journal of Medical Microbiology, 2014, 4, 153-160
Published Online August 2014 in SciRes. http://www.scirp.org/journal/ojmm
http://dx.doi.org/10.4236/ojmm.2014.43018

Detection of the Mex Efflux Pumps in *Pseudomonas aeruginosa* by Using a Combined Resistance-Phenotypic Markers and Multiplex RT-PCR

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Received 16 June 2014; revised 14 July 2014; accepted 14 August 2014

Abstract

The aim of this study was to detect the expression of 4 clinically-important efflux pumps in the Resistance-Nodulation-Cell Division (RND) family including MexAB-OprM, MexXY, MexCD-OprJ and MexEF-OprN in *Pseudomonas aeruginosa* using a combination of resistance-phenotypic markers and multiplex RT-PCR (mRT-PCR). The antibiotic substrates specific for each Mex systems were used as phenotypic markers including carbenicillin, MexAB-OprM, erythromycin, MexCD-OprJ, norfloxacin and imipenem, MexEF-OprN and gentamicin, MexXY-OprM. The methods were validated with reference strains with known genotypes of the Mex systems and the potential applicability in clinical practice was tested with clinical isolates. The results for the reference strains support that the combination of resistance phenotype and mRT-PCR is a potential-attractive method for diagnosis of efflux-mediated resistance in *P. aeruginosa*. Further development to make it more practical for clinical use and study in a larger number of clinical isolates is required.

Keywords

Multidrug Efflux Pumps, Multiplex RT-PCR, *Pseudomonas aeruginosa*, Resistance-Phenotypic Marker

1. Introduction

Pseudomonas aeruginosa, a common cause of nosocomia infections is infamous for its resistance to multiple

How to cite this paper: Poonsuk, K. and Chuanchuen, R. (2014) Detection of the Mex Efflux Pumps in *Pseudomonas aeruginosa* by Using a Combined Resistance-Phenotypic Markers and Multiplex RT-PCR. *Open Journal of Medical Microbiology*, **4**, 153-160. http://dx.doi.org/10.4236/ojmm.2014.43018

drugs [1] that is mainly attributed to the synergy between the low outer membrane permeability and the expression of multidrug efflux systems, particularly in the Resistance-Nodulation-Cell Division (RND) family [2]. Most RND-type drug efflux operons are chromosomally encoded. It is now generally accepted that the RND multidrug efflux systems function as tripartite systems consisting of a cytoplasmic membrane-associated RND transporter (e.g. MexB, MexD, MexF, MexY), periplasmic membrane fusion protein (MFP) e.g. MexA, MexC, MexE and MexX and an outer membrane protein (e.g. OprM, OprJ, and OprN) [3]. The *P. aeruginosa* genome contains at least 12 structural genes for the RND efflux systems, of which four are clinically-important (*i.e.* MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY) [2]. Due to their constitutive expression, MexAB-OprM and MexXY contribute to intrinsic resistance to many antibiotics [2]. MexCD-OprJ and MexEF-OprN do not express wild-type cells, but can overexpress after the acquisition of regulatory mutations, resulting in acquired multidrug resistance [4] [5]. Coexpression of Mex systems has been reported in the *P. aeruginosa* clinical isolates where its variable impact on antibiotic susceptibility has been observed [6]-[8].

Diagnosis of efflux-mediated resistance generates data that is helpful for both routine clinical analysis (e.g. rationalizing the antibiotic selection and dose) and epidemiological studies (e.g. monitoring the existing and prevalent resistance mechanisms) [9]. Efflux pump inhibitors (EPIs) have been under investigation as an alternative to the development of new antibiotics for treatment of *P. aeruginosa* infection [10]. As yet, no EPIs are approved for clinical use. However, detection methods for efflux-mediated resistance should be concurrently developed in preparation for the new treatment protocol. Phenotypic-based methods usually yield vague outcomes due to the possible existence of other resistance mechanisms, the simultaneous over expression of variable Mexs and the difficulty in assessing the pumps conferring low or moderate resistance level [9]. Detection of the Mex systems has long relied on western blot analysis using monoclonal or polyclonal antibodies. The technique is complicated and time consuming and the antibodies specific for the Mex protein are not commercially available. Quantitative realtime RT-PCR (realtime qRT-PCR) is a rapid method for measuring gene expression. However, many probes are required for simultaneous detection of many Mex genes, resulting in increased cost. In a previous study, realtime qRT-PCR was applied to measure the Mex expression level but limited to that of only 2 Mex systems (*i.e.* MexAB-OprM and MexXY) [11]. In contrast, quantitative RT-PCR (qRT-PCR) is less expensive but laborious. The disadvantage may be resolved by concomitant detecting of multiple Mex genes using multiplex-qRT-PCR (mRT-PCR). The latter allows us to easily observe the amplification products and is feasible in the laboratory where a realtime PCR machine is not available. Recently, the combined phenotypic and genotypic methods were used for detecting the expression of all 4 clinically-important Mex systems [9]. However, two different PCR-based methods were used for detecting each two systems in the same sample. So far, none of these new diagnostic methods have been commercially available.

In this study, we have combined antibiotic-resistance-phenotypic markers and mRT-PCR for detecting the expression of MexAB-OprM, MexXY, MexCD-OprJ and MexEF-OprN in *P. aeruginosa*. The methods were validated in reference strains with known genotypes of the Mex systems and the potential applicability in clinical practice was tested with clinical isolates.

2. Methods

2.1. Bacterial Isolates and Growth Conditions

The *P. aeruginosa* reference strains including PAO1 [12], PAO200, PAO200-2, PAO238, PAO7H1A, PAO255 [13], PAO267 and PAO280 [13] [14] and clinical isolates used in this study are listed in **Table 2** and **Table 3**. The reference strains were selected as carrying known-RND efflux pumps expressed. The clinical isolates were randomly selected from our strain collection. Each of them was isolated from different patients admitted at Siriraj Hospital, Bangkok, Thailand in previous studies [15]. The clonality of the clinical isolates was examined by using ERIC PCR [16] and all were confirmed to be nonrepetitive strains (data not shown). All the *P. aeruginosa* strains were grown on Luria Bertani (LB) agar, LB broth (Difco, BD Diagnostic Systems, MD, USA) or in Mueller-Hinton broth (MHB; Difco). All the bacterial cultures in LB broth were incubated at 37˚C, with agitation at 120 rpm and under aerobic condition for 12 h. All the bacteria in LB agar and MHB were grown with aeration at the same temperature and period of time.

2.2. Antimicrobial Susceptibility Testing

MICs of antibiotics tested including carbapenem (Car), erythromycin (Ery), imipenem (Imi), norfloxacin (Nor)

and gentamicin (Gen), were determined by using two-fold both microdilution method in the presence and absence of Phe-Arg-*β*-naphthylamide (PA*β*N), a broad spectrum EPI, at the concentration of 50 µg/ml (Sigma Aldrich, St. Louis, MO, USA) [17]. All antibiotics were purchased from Sigma Aldrich. *P. aeruginosa* ATCC 27853 [18] and wild-type PAO1 were used as control strains.

2.3. RNA Extraction and cDNA Synthesis

The *P. aeruginosa* cells grown in LB were harvested at 12 h of growth (A_{540}~5.5) by centrifugation. The cells were immediately used for RNA extraction using Total RNA Extraction Mini Kit (RBC Bioscience, New Taipei City, Taiwan) and subsequently treated with RNase-free DNaseI (Fermentas®, Mainz, Germany) as suggested by the manufacture's instruction. The absence of genomic DNA residuals was determined by PCR. Synthesis of cDNA was performed by reverse transcription using ImProm-II™ Reverse Transcriptase (Promega, WI, USA). Each 5 µl RNA-primer mixture contained 0.5 µg of free DNA-RNA, 10 pmol of each reverse primer (mexBMRT down, mexDRTdown, mexFRTdown and mexYMRTdown). The mixture was incubated at 70˚C for 5 min, quickly chilled at 4˚C for 5 min and hold on ice. The reverse transcription PCR reaction was performed in a 20 µl volume containing 6 µl of the RNA-primer mixture, 4 µl of Improm-II™ 5X Reaction Buffer, 2 µl of 25 mM $MgCl_2$, 1 µl of dNTPs (10 mM each), 1 µl of Improm-II™ Reverse Transcriptase and nuclease free water added to 20 µl. The PCR cycles were as follows: annealing for 5 min at 25˚C, extension for 45 min at 45˚C and heat-inactivation for 15 min at 70˚C. The cDNA was stored at −20˚C until used. All the primers were designed by Primer3 software available at http://bioinfo.ut.ee/primer3-0.4.0/primer3/ (**Table 1**).

2.4. Multiplex RT-PCR

cDNA from each bacterial strain was individually used as DNA template in mRT-PCR, as well as the mixture of cDNA from all reference strains. All the mRT-PCR reactions were carried out in a 30 µl volume containing 5 µl cDNA (100 - 2000 ng/ml), 10 pmol of each primer, 15 µl of KAPATaq ReadyMix DNA polymerase (Kapabiosystems, MA, USA) and nuclease free water added to 30 µl. The PCR amplication was performed according to the following cycles: one predenaturation for 5 min at 95˚C and 30 cycles of denaturation for 45 s at 95˚C, annealing for 45 s at 54˚C and extension for 30 s at 72˚C, followed by final extension for 10 min at 72˚C.

2.5. PCR amplification and DNA Sequencing

All the conventional PCR amplifications were conducted using KAPA Taq ReadyMix (KAPAbiosystem) according to the manufacturer's instruction. The PCR amplicons from either conventional PCR or mRT-PCR were gel-purified using Nucleospin® ExtractII (Mccherey-Nagel, Düren, Germany) and submitted for sequencing with the PCR primers at 1st BASE Pte Ltd. (Gemini Singapore Science ParkII, Singapore). The DNA sequences obtained were compared with the corresponding sequences of PAO1 available at the *Pseudomonas* Genome Project [19].

3. Results

3.1. Resistance Phenotypes Associated with the Mex Efflux Pumps

Five antibiotics were used as resistance-phenotypic markers for 4 clinically-important Mex systems tested in this study: carbenicillin, MexAB-OprM; erythromycin, MexCD-OprJ; norfloxacin and imipenem, MexEF-OprN and gentamicin, MexXY-OprM. The MIC values for these antibiotic markers in the presence and absence of PA*β*N are shown in **Table 2** and **Table 3**. For the reference strains overexpressing a Mex pump, the addition of Phenylalanine-Arginine *β*-Naphthylamide (PA*β*N) reduced the MIC values for the corresponding antibiotic markers 2 to 128 folds. The lowest-reduction (2 fold) was observed for imipenem MIC in PAO7H1A overexpressing MexEF-OprN. Most of the clinical isolates exhibited higher resistance level to all antibiotics tested than PAO1 did. PA*β*N, also called MC-207,110, is a peptidomimetic compound that functions as a broad-spectrum EPI. At the concentration above 16 µg/mL, PA*β*N additionally permeabilizes membranes in MexAB-OprM deficient *P. aeruginosa* mutants [20].

3.2. Expression of the Mex Efflux Pumps Determined by mRT-PCR

For the reference strains, the expression of the Mex efflux pumps is shown in **Table 2**. When the mixture of cDNA

Table 1. Primers used in this study.

Target	Primer	Sequence (5'-3')	Amplicon size (bp)	Source
mexB	mexBMRTup	5'-ACTTCTTCAGCTTCAAGGAC-3'	155	This study
	mexBMRTdown	5'-GAGCATGAGGAACTTGTTG-3'		
mexD	mexDRTup	5'-CTACCCTGGTGAAACAGC-3'	250	This study
	mexDRTdown	5'-AGCAGGTACATCACCATCA-3'		
mexF	mexFRTup	5'-CATCGAGATCTCCAACCT-3'	350	This study
	mexFRTdown	5'-GTTCTCCACCACCACGAT-3'		
mexY	mexYMRTup	5'-GCTACAACATCCCCTATGAC-3'	445	This study
	mexYMRTdown	5'-AACTGGCGGTAGATGTTG-3'		

Table 2. Phenotypic and genotypic properties of the *P. aeruginosa* reference strains.

Strain	Relevant genotype	Expressed efflux	Antibiotic marker	MIC (μg/ml)			Efflux determined by Multiplex RT-PCR
				−PAβN	+PAβN	Reduction fold	
PAO1	Wild-type	MexAB-OprM	Carbenicillin	128	16	8	MexAB-OprM
PAO200	PAO1Δ(*mexAB-oprM*)	None		1	1	0	None
PAO1	Wild-type	MexAB-OprM	Erythromycin	512	256	2	Wild-type
PAO200	PAO1Δ(*mexAB-oprM*)	None		8	8	-	None
PAO200-2	PAO200*nfxB*	MexCD-OprJ		512	4	128	MexCD-OprJ
PAO238	PAO200-2Δ(*mexCD-oprJ*)	None		32	4	8	None
PAO7H1A	PAO7H[a]Δ(*mexAB-oprM*)	MexEF-OprN	Imipenem	8	4	2	MexEF-OprN
PAO255	PAO7H1AΔ(*mexEF-oprN*)	None		1	0.5	2	None
PAO7H1A	PAO7HΔ(*mexAB-oprM*)	MexEF-OprN	Norfloxacin	2	0.06	33	MexEF-OprN
PAO255	PAO7H1AΔ(*mexEF-oprN*)	None		<0.06	<0.06	-	None
PAO267	PAO3579[b] with Δ(*mexAB-oprM*)	MexXY	Gentamicin	2	0.03	64	MexXY
PAO280	PAO267 with Δ(*mexXY*)	None		2	0.25	8	None

[a]PAO7H, overproduced Mex EF-OprN [21], [b]PAO3579, PAO1Δ(*amr*) [22].

Table 3. Antimicrobial susceptibility and expression of the RND efflux determined by Multiplex RT-PCR in the *P. aeruginosa* Clinical isolates.

Clinical isolate	Relevant genotype	MIC (μg/ml)										Efflux determined by Multiplex RT-PCR
		Car		Ery		Imi		Nor		Gen		
		−PAβN	+PAβN	−PAβN	+PAβN	−PAβN	+PAβN	−PAβN	+PAβN	−PAβN	+PAβN	
PAJ114	-	16384	8192	256	32	256	32	64	16	512	256	AB
PAJ128	-	16384	4096	256	64	256	32	128	16	512	256	AB, CD, XY
PAJ147	-	16384	4096	256	4	64	32	128	16	512	256	AB, CD, XY
PAJ197	-	16384	8192	256	64	>256	16	64	32	256	128	AB, EF, XY
PAJ207	-	128	32	256	64	>256	256	8	1	16	8	AB, EF, XY
PAJ212	-	8192	1024	256	64	256	32	128	16	>16384	1024	AB, XY
PAJ215	-	8192	4092	256	64	256	32	16	16	>16384	4092	AB, XY

Not known. AB, MexAB-OprM; CD, MexCD-OprJ; EF, MexEF-OprN, XY, MexXY-OprM.

from all the reference strains was used for template, PCR amplicons of all 4 Mex systems was obtained (**Figure 1**) and DNA sequencing analyses confirmed their specificity. By using mRT-PCR, MexB, MexD, MexF and MexY expression was detected in PAO1, PAO200-2, PAO7H1A and PAO267, respectively. None of the Mex expression was observed in PAO200, PAO238, PAO255 and PAO280 that are null mutant derivatives.

All the clinical isolates produced MexAB-OprM and MexXY as determined by mRT-PCR. Four isolates expressed 3 Mex systems including PAJ128 and PAJ147 (overexpressing MexAB-OprM, MexCD-OprJ and MexXY) and PAJ197 and PAJ207 (overexpressing MexAB-OprM, MexEF-OprN and MexXY). All the isolates were resistant to imipenem and norfloxacin but MexEF-OprN expression was observed in 2 isolates (PAJ197 and

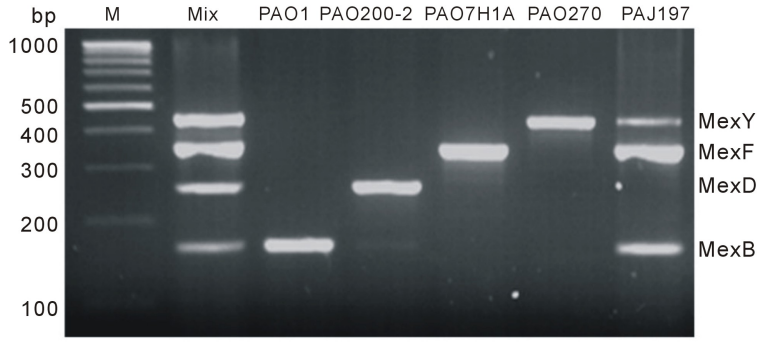

Figure 1. PCR amplicons of 4 clinically important Mex systems generated by RT-PCR. Lane 1) M, molecular weight marker; Lane 2) Mix, The mixture of cDNA from PAO1, PAO200-2, PAO7H1A and PAO27 was used as template. The size of *mexB* (155 bp), *mexD* (250 bp), *mexF* (350 bp) and *mexY* (445 bp). Lane 3 - 6) band for *mexB* (155 bp), *mexD* (250 bp), *mexF* (350 bp) and *mexY* (445 bp) PAO1 overexpressing MexB; PAO200-2 overexpressing MexD; PAO7H1A overexpressing MexF and PAO270 overexpressing MexY respectively. Lane 7, PAJ197 a clinical isolate overexpressing MexB/MexF and MexY.

PAJ207). The similar result was observed for erythromycin resistance.

4. Discussion

For phenotype detection, the antibiotics used for phenotypic markers are specific substrates for each of 4 clinically-important Mex systems based on previous studies [23]-[26]. For MexEF-OprN, imipenem was also included as an indirect indicator of pump as previously suggested [9]. In the up-regulated MexEF-OprN mutant strain, resistance to carbapenems is a result of down-regulated OprD that happens concomitantly with the elevated-expression of MexEF-OprN [21] [27]. In the present study, the addition of PAβN caused a 2 fold-reduction of imipenem MIC in both PAO7H1A overexpressing only MexEF-OprN and its isogenic-null mutant PAO255. Similarly, PAβN also reduced the imipenem MIC in all clinical isolates either with or without MexEF-OprN expression. These observations suggest the possible existence of uncharacterized Mex systems that are also inhibited by PAβN in these strains. However, the OprD expression and its actual involvement of MexEF-OprN in the MexEF-OprN-overespressing isolates were not examined. Several studies showed that the synchronized expression and carbapenem resistance was not always observed in clinical isolates [28]. It is evident by our observation that some isolates with high imipenem MICs (e.g. PAJ128 and PAJ147) did not produce MexEF-OprN. Taken together, these data confirmed that imipenem is not a good-indirect phenotypic marker for MexEF-OprN.

In the laboratory reference strains, a good agreement was observed between the RND-efflux genotype and the Mex expression determined by mRT-PCR in all the reference strains (**Table 2**). For example, only expression of MexD was detected in PAO200-2 that is a spontaneous *nfxB* derivative of PAO200. The good correlation was also identified between resistance phenotype and the Mex expression. For the instance, PAO1 constitutively producing MexAB-OprM exhibited high carbenicillin MIC. PAO200-2 overexpressing MexCD-OprJ was highly resistant to erythromycin.

Unlike the laboratory reference strains, clinical isolates have diverse genetic backgrounds, resulting in diverse phenotypes. Therefore, it cannot simply use resistance phenotype to predict the expressed-Mex efflux pumps. The discrepancy may be overcome by increasing the number of antibiotic markers to cover all the possible Mex systems. This will markedly cause increasing cost and thus, decreasing the attraction of the method. The addition of a specific Mex inhibitor is of interest but such inhibitors are still not commercially available [9]. Transcription of MexB and MexY was detected in all the clinical isolates, is in agreement with the fact that these efflux systems are always expressed at basal level. The good correlation of resistance phenotype and MexCD-OprJ was observed in two isolates (PAJ128 and PAJ147) and that of MexEF-OprN was observed in PAJ197 and PAJ207. This may not be surprising because MexCD-OprJ and MexEF-OprN are expressed in regulatory mu-

tants and their contribution to antibiotic markers may vary. The addition of PAβN revealed that the contribution of the Mex systems in resistance level varied in clinical isolates and suggested the existence of other enzymatic or non-enzymatic resistance mechanisms. The marginal effect (*i.e.* 2 fold reduction) of the Mex systems on antibiotic susceptibility was observed in some isolates. It could prevent antibiotics from reaching their optimal concentrations in target organs, especially where the antibiotic concentrations are hindered (e.g. in pus, biofilms, lung tissues), and therefore is still of clinical importance [15].

A comment could be made that the usefulness of mRT-PCR may be less in comparison to realtime qRT-PCR because the Mex expression level was not quantified. Several studies showed no correlation between the level of transcription and resistance in *P. aeruginosa* clinical isolates from either animals or humans [29] [30]. Therefore, the measurement of expression level is not always essential for routine diagnosis. Importantly, mRT-PCR could be easily performed in most clinical laboratories, especially those without a sophisticated realtime PCR machine. Still, it cannot be disputed that MIC determination is a gold standard method for assessing susceptibility of *P. aeruginosa* before choosing antibiotic treatment. For better treatment regimen, mRT-PCR will allow optimal antibiotic choices, especially antibiotics available for use in combination with EPIs.

5. Conclusion

The results in this study support that the combination of resistance phenotype and mRT-PCR is a potential-attractive method for diagnosis of efflux-mediated resistance in *P. aeruginosa*. mRT-PCR is rapid and specific for detection of the Mex systems. However, further development to make it more practical for clinical use and study in a larger number of clinical isolates is still required. The appropriate antibiotics that can be specifically used for the Mex pumps need to be explored. Although the knowledge from this study requires more research before the application in clinical analysis, it is a useful tool for epidemiological studies of the prevalent Mex systems without delay.

Acknowledgements

We thank Professor Herbert P. Schweizer, Department of Microbiology, Immunology, and Pathology, Colorado State University, USA for reference strain providing. This work was supported by the Research Grant for Mid-Career University Faculty RMU5380035 co-funded by Thailand Research Fund (TRF), the Commission on Higher Education, the Faculty of Veterinary Science, and Chulalongkorn University. KP is the recipient of the Royal Golden Jubilee Ph.D. program PHD/0014/2552, co-funded by TRF and Chulalongkorn University.

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