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Original Article

The Multidrug-Resistant *Pseudomonas aeruginosa* Clinical Isolates from Dogs and Cats Expressed Three Multidrug Efflux Systems Simultaneously

Kanchana Poonsuk[1] Rungtip Chuanchuen[1*]

Abstract

The objective of this study was to examine the expression of 4 clinically-important Mex systems including MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY in the *Pseudomonas aeruginosa* clinical isolates from dogs and cats. The isolates exhibited high level of resistance to multiple antibiotics clinically important. All of them simultaneously overexpressed up to three different Mex systems, including MexAB-OprM, MexEF-OprN and MexXY as determined by RT-PCR. None of the isolates overexpressed MexCD-OprJ. Expression of *mexF* was measured by using quantitative real-time RT-PCR. Transcription level of *mexF* varied (i.e. 4-219 fold) but was at least 4 fold higher than that of PAO1. DNA sequence analysis of *mexT* suggested the existence of uncharacterized regulatory mechanism (s) of MexEF-OprN expression besides MexT. The results underscored the contribution of Mex systems in multidrug resistance phenotype of the *P. aeruginosa* clinical isolates from dogs and cats.

Keywords: cat, dog, multidrug efflux system, multidrug resistance, *Pseudomonas aeruginosa*

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Introduction

Pseudomonas aeruginosa is an important opportunistic pathogen that inflicts diseases in both humans and animals. In dogs and cats, this pathogen is notoriously known as a common cause of chronic and recurrent infections, most notably otitis externa/media, pyoderma and urinary tract infection (Petersen et al., 2002; Gatoria et al., 2006; Hariharan et al., 2006). Chronic *Pseudomonas* infections usually require constant treatment with different antibiotic mixtures. However, *P. aeruginosa* is infamous for its multidrug resistance phenotype that is mainly attributed to the synergy between low outer membrane permeability and expression of multidrug efflux systems, particularly those in the Resistance-Nodulation-Cell Division (RND) family (Aksamit, 1993; Lister et al., 2009).

The *P. aeruginosa* genome contains up to 12 structural genes for the RND efflux systems, of which four are clinically important (i.e. MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY) (Lister et al., 2009). 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) (Poole and Srikumar, 2001). MexAB-OprM and MexXY are constitutively expressed and contribute to intrinsic resistance to many antibiotics (Lister et al., 2009). MexXY is the only Mex system that mediates natural resistance to aminoglycosides (AMG) and additionally extrudes antibiotic substrates including tetracycline, macrolides and fluoroquinolones (Masuda et al., 2000). MexCD-OprJ is normally quiescent in wild-type *P. aeruginosa* and is upregulated in the mutant strains with impaired regulatory genes, *nfxB* (Schweizer, 1998). Its antibiotic substrates include some β-lactams, fluoroquinolones, macrolides and tetracycline (Morita et al., 2001). Correspondingly, MexEF-OprN is typically silent in wild-type cells and overproduced in the *nfxC*-type mutants. This Mex system confers resistance to several antibiotics, e.g. imipenem, fluoroquinolones, chloramphenicol, trimethroprim and tetracycline (Kohler et al., 1997).

Bacterial efflux pump inhibitors (EPIs) have been researched and become promising therapeutic agents (Lomovskaya and Watkins, 2001). The molecules are potential for use in combination with antibiotic therapy and expected to restore the activity of standard antibiotics by increasing the intracellular concentration of antibiotics that are expelled by the Mex pumps. The EPI-antibiotic combination is anticipated to be a novel medical treatment options for infections with *P. aeruginosa* in either humans or animals (Tegos et al., 2002). Therefore, the need to understand the role and functions of Mex systems has been elevated to accomplish new therapeutic efforts. Up to date, contribution of the Mex pumps has been extensively studied in the *P. aeruginosa* human isolates. Previous studies showed coexpression of Mex systems in the human isolates and their variable impact on antibiotic susceptibility has been observed (Aendekerk et al., 2002; Wolter et al., 2004; Sevillano et al., 2006). In contrast, knowledge of the Mex systems in the animal isolates is still limited. We previously described the function and involvement of the MexXY efflux pump in AMG resistance in the clinical isolates from cow mastitis (Chuanchuen et al., 2008), dogs and cats (Poonsuk et al., 2014). In this study, we aimed to examine the expression of 4 clinically important Mex systems including MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY in the MDR *P. aeruginosa* clinical isolates from dogs and cats.

Materials and Methods

Bacterial isolates and growth conditions: Ten *P. aeruginosa* clinical isolates from dogs and cats were included in this study (Table 1). All were characterized for the expression of MexXY in our previous study (Poonsuk and Chuanchuen, 2012). All isolates were originated from samples that were collected from dogs and cats at Small Animal Hospital during 2005-2010 and submitted for bacterial isolation at the VDL. A single *P. aeruginosa* colony was collected from each positive sample. However, genetic relatedness of the isolates was not examined. *P. aeruginosa* strain PAO1, constitutively produces MexAB-OprM, was used as a reference strain in gene expression experiments (Watson and Holloway, 1978). All the *P. aeruginosa* strains were grown on Luria Bertani (LB) broth and LB agar (Difco, BD Diagnostic Systems, MD, USA). For antimicrobial susceptibility testing, the isolates were grown on Mueller-Hinton agar (MHA; Difco) and in Mueller-Hinton broth (MHB; Difco) with adjusted concentrations of Ca^{2+} and Mg^{2+}.

Antimicrobial susceptibility testing: Minimum Inhibitory Concentrations (MICs) of 17 antimicrobials were determined by using two-fold agar dilution and broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013). The antibiotics included carbenicillin (Car), ciprofloxacin (Cip), tetracycline (Tet), ceftaxidime (Ctz), trimethoprim (Tri), chloramphenicol (Chp), amikacin (Amk), gentamicin (Gen), kanamycin (Kan), neomycin (Neo), streptomycin (Str), spectinomycin (Spc), tobramycin (Tob), erythromycin (Ery), imipenem (Imp), sulfonamide (Sul) and ticarcillin (Tic). CLSI-MIC breakpoints were used when available (Table 3). The CLSI interpretive breakpoints for the Enterobacteriaceae and those in published data were used for antimicrobials that lacked the CLSI breakpoints, i.e. streptomycin, neomycin and tobramycin (Rubin et al., 2008). Experiments were performed in triplicate and repeated independently twice. *P. aeruginosa* ATCC 27853 and wild-type PAO1 were used as quality control organisms.

PCR and DNA sequencing: All the primer pairs used in this study are listed in Table 2. PCR amplifications were performed using KAPATaq ReadyMix DNA polymerase (Kapabiosystems, Boston, MA, USA) as described in the manufacturer's protocol. *mexT* gene was PCR-amplified using primers mexT1up and mexT2down. PCR products were purified directly or from agarose gels using Nucleospin ExtractII

(Mccherey-Nagel, Düren, Germany) and submitted for nucleotide sequencing at 1stBASE Pte, Ltd (Singapore Science Park II, Singapore). DNA sequencing results were compared to the *mexT* sequence of wild-type strain, PAO1 available at the *Pseudomonas* Genome Project (http://pseudomonas.com).

Reverse transcription (RT)-PCR: All isolates were screened for the transcription of MexB, MexD and MexF using conventional RT-PCR. Total RNA was extracted using Total RNA Extraction Mini kit (RBC Bioscience, Taipei, Taiwan) and treated with RNase-free DNaseI (Fermentus, Ontario, Canada). Single stranded-cDNA was synthesized from one-μg DNase treated RNA using ImProm-II™ Reverse Transcriptase (Promega, Madison, WI, USA) with reverse primers specific to *mexB* (mexBRTup/mexBRT down), *mexD* (mexDRTup/mexDRTdown), *mexF* (mexFRTup/mexFRTdown) and *mexY* (mexYRTup/mexYRTdown). The cDNA was used as the template for PCR amplification using the specific primer pairs as described above.

Table 1 Bacterial strains used in this study

Strain	Source	Reference
PAO1	Wild-type	Watson and Holloway (1978)
PAJ227	Urine from cat	Poonsuk and Chuanchuen (2012)
PAJ228	Feline nasal cavity	Poonsuk and Chuanchuen (2012)
PAJ229	Urine from cat	Poonsuk and Chuanchuen (2012)
PAJ230	Feline nasal cavity	Poonsuk and Chuanchuen (2012)
PAJ232	Otitis ear in dog	Poonsuk and Chuanchuen (2012)
PAJ233	Pus from wound in cat	Poonsuk and Chuanchuen (2012)
PAJ235	Urine from dog	Poonsuk and Chuanchuen (2012)
PAJ239	Pus from wound in cat	Poonsuk and Chuanchuen (2012)
PAJ240	Otitis ear in dog	Poonsuk and Chuanchuen (2012)
PAJ245	Pus from wound in cat	Poonsuk and Chuanchuen (2012)

Table 2 Primers used in this study

Gene	Primer	Sequence (5´-3´)	Reference
mexB	mexBRTup	ATCTACCGGCAGTTCTCC	Poonsuk et al. (2014)
	mexBRTdown	CGATCACCACGTAGATCAG	Poonsuk et al. (2014)
mexD	mexDRTup	CTACCCTGGTGAAACAGC	Poonsuk et al. (2014)
	mexDRTdowm	AGCAGGTACATCACCATCA	Poonsuk et al. (2014)
mexF	mexFRTup	CATCGAGATCTCCAACCT	Poonsuk et al. (2014)
	mexFRTdown	GTTCTCCACCACCACGAT	Poonsuk et al. (2014)
mexY	mexYRTup	AGCTACAACATCCCCTA	Chuanchuen et al. (2008)
	mexYRTdown	AGCACGTTGATCGAGAAG	Chuanchuen et al. (2008)
mexT	mexT1up	CAGTTCGAAGCCGAGACC	Poonsuk et al. (2014)
	mexT2down	AGCGGTTGTTCGATGACTTC	Poonsuk et al. (2014)
rpsL	rpsLrealtimeup	CGGCACTGCGTAAGGTATG	Chuanchuen et al. (2008)
	rpslrealtimedown	CCCGGAAGGTCCTTTACACG	Chuanchuen et al. (2008)

Quantitative real-time PCR (qRT-PCR): Expression level of *mexF* was quantified by qRT-PCR as previously described with some modifications (Chuanchuen et al., 2008; Islam et al., 2009). One μg of total RNA was used to synthesize cDNA as described above. The cDNA obtained was quantified using KAPA SYBR® FAST qPCR kit (Kapabiosystems). PCR assays were performed in triplicate. The average cDNA copy numbers of *mexT* was estimated using Ct values from two separate experiments (SD< 0.1). The average *mexF* cDNA copy number was normalized with that of *rpsL*, a house keeping gene serving as the internal control. The transcription level of *mexF* was compared to PAO1 and presented as fold change ratios.

Results

***Antimicrobial susceptibility*:** All the isolates exhibited resistance to at least 9 antimicrobial agents tested (Table 3). All were resistant to tetracycline, trimethoprim, chloramphenicol, streptomycin, spectinomycin and erythromycin. High MIC level (≥256 μg/ml) was observed for trimethoprim, chloramphenicol, sulphonamide and spectinomycin in all isolates (data not shown). All the isolates but one (i.e. PAJ237) were susceptible to imipenem.

Expression of Mex systems: The results from conventional RT-PCR showed that all the clinical isolates expressed MexB, MexF and MexY (Table 3). None were found to produce MexD. Transcription level of *mexF* was determined with qRT-PCR and was found to be at least 4-fold higher than that of PAO1 (i.e. 4-219 fold) in all the isolates (Table 3). The MexF expression level was higher than 50-fold in most isolates (i.e. PAJ227, PAJ230, PAJ233, PAJ235 and PAJ240). Among these isolates, PAJ235 produced the highest MexF, 219-fold greater than that of PAO1.

Mutation (s) in mexT: Four clinical isolates were selected for nucleotide sequencing analysis of *mexT*. Two of them were the clinical isolates with high MexF expression level (i.e. PAJ235, 219 fold and PAJ240, 75 fold). The others were those with lowest-MexF production (i.e. PAJ228, 8.5 fold and PAJ229, 4 fold). In comparison to *mexT* sequence of PAO1, all four clinical isolates tested lacked insertion of 8 nucleotides (5´cggccagc3´) between nucleotide positions 104 to 105 of *mexT*. All additionally had a single point mutation

that is a replacement of T385 with A leading to a Phe-129- Ile substitution in MexT. The lowest expression level (4 fold) was observed in PAJ229.

Discussion

In this study, 10 *P. aeruginosa* clinical isolates originated from dogs and cats were assessed for antimicrobial resistance and expression of Mex systems. However, the isolates could not be traced back to the exact antibiotic exposure due to the unsystematic recording of the antibiotic therapy. All the isolates were resistant to multiple drugs of different classes. Such simultaneous decreased susceptibilities to a variety of antibiotics were indicative of the expression of one or many nonenzymatic resistance mechanism in these isolates, including Mex systems of the RND family.

One of the major findings was that three Mex systems including MexAB-oprM, MexEF-OprN and MexXY expressed simultaneously in the *P. aerginosa* isolates obtained from dogs and cats. This is consistent with a previous study reporting coexpression of two Mex systems, i.e. MexAB-OprM/MexXY and MexAB-OprM/MexEF-OprN in the isolates from canine ears (Beinlich et al., 2001). The concomitant expression of Mex systems has been previously shown in human clinical isolates in several studies, for example, MexXY/MexJK (Hocquet et al., 2006), MexAB-OprM/MexXY (Hocquet et al., 2006; Llanes et al., 2011), and MexAB-OprM/MexCD-OprJ (Llanes et al., 2011). Recently, our study reported the simultaneous expression of up to four Mex systems in a *P. aeruginosa* clinical isolate from non-CF patients (Poonsuk et al., 2014). Taken together, these results support the wide expression of the RND-multidrug efflux systems and their contribution to multidrug resistance in the *P. aruginosa* clinical isolates from humans and animals.

All the animal isolates in the present study expressed MexAB-OprM, in agreement with a previous study of the clinical isolates from various animal sources (Beinlich et al., 2001). This confirms the constitutive expression of MexAB-OprM in the *P. aeruginosa* animal isolates and its role in intrinsic resistance to antimicrobials. The expression of MexXY was observed in all the isolates in this collection as previously determined (Poonsuk et al., 2014). It was shown that MexXY played a role in AMG resistance in the dog and cat isolates. However, MexXY expression alone did not fully account for AMG resistance observed, suggesting the existence of additional uncharacterized AMG resistance mechanisms (Poonsuk et al., 2014).

Table 3 Phenotypic and genetic characteristics of *P. aeruginosa* clinical isolates from dogs and cats (n=10)

Strain	Mex expression				Transcription level of MexF	*mexT* mutation[a]	MIC (μg/ml)				Resistance pattern
	MexB	MexD	MexF	MexY			Imp	Chp	Tri	Tet	
PAO1	+	-	-	+	1	(+)	1	32	256	32	Chp-Tri-Tet
PAJ 227	+	-	+	+	95	ND	1	>256	>256	64	Car-Tet-Tri-Chp-Str-Spc-Ery-Sul-Tic
PAJ 228	+	-	+	+	8.5	(-)[b]	1	>256	>256	128	Car-Tet-Tri-Chp-Gen-Kan-Str-Spc-Ery-Sul-Tic
PAJ 229	+	-	+	+	4	(-)[b]	2	>256	>256	64	Tet-Tri-Chp-Gen-Kan-Neo-Str-Spc-Ery-Sul-Tic
PAJ 230	+	-	+	+	48.5	ND	1	>256	>256	128	Cip-Tet-Tri-Chp-Kan-Neo-Str-Spc-Ery-Sul-Tic
PAJ 232	+	-	+	+	50.5	ND	2	256	>256	64	Tet-Tri-Chp-Str-Spc-Tob-Ery-Sul-Tic
PAJ 233	+	-	+	+	52.5	ND	0.5	256	>256	64	Tet-Tri-Chp-Kan-Neo-Str-Spc-Ery-Sul-Tic
PAJ 235	+	-	+	+	219	(-)[b]	1	>256	>256	64	Tet-Tri-Chp-Kan-Neo-Str-Spc-Ery-Sul-Tic
PAJ 237	+	-	+	+	10.5	ND	16	>256	>256	>256	Tet-Tri-Chp-Gen-Kan-Neo-Str-Spc-Ery-Imp-Sul-Tic
PAJ 239	+	-	+	+	39	ND	2	256	>256	64	Tet-Tri-Chp-Kan-Str-Spc-Ery-Sul-Tic
PAJ 240	+	-	+	+	75	(-)[b]	1	256	>256	64	Car-Tet-Tri-Chp-Kan-Neo-Str-Spc-Ery-Sul-Tic
Breakpoint							16	32	4	16	

[a](+), with or (-), without insertion of 8 nucleotides (5'cggccagc3')
[b]with an additional mutation Phe(TTC)-129-Ile(ATC)
ND= not determined

Of particular interest is the expression of MexEF-OprN in all the *P. aeruginosa* isolates from dogs and cats. This is inconsistent with a previous study showing that only 1 of 12 *P. aeruginosa* from various animals expressed this efflux system (Beinlich et al., 2001). This discrepancy could be associated with the genetic diversity of the *P. aeruginosa* isolates from different geographical region and also different antibiotic exposure in different hospital settings. As expected, the isolates in this study were highly resistant to chloramphenicol, trimethoprim and tetracycline, which are specific substrates of MexEF-

OprN (Kohler et al., 1997). In contrast, only one isolate (i.e. PAJ237) was resistant to imipenem. Previous studies demonstrated coregulation of MexEF-OprN and an outer membrane protein, OprD. The coregulation is mediated by MexT and results in up-regulated MexEF-OprN and down-regulated OprD (Kohler et al., 1997). Mutants with up-regulated MexEF-OprN and down-regulated OprD were exclusively resistant to carbapenems, including imipenem. In this case, the concerted decrease in OprD (not overexpressed MexEF-OprN) significantly contributes to imipenem resistance in the *nfxC*-type mutants (Kohler et al., 1997; Ochs et al., 1999). However, the expression of OprD was not pursued in this study.

MexEF-OprN is a distinctive Mex pump. This is because it is the only Mex system, of which expression is regulated by a transcriptional activator of LysR-type family, MexT, encoded by *mexT* located upstream of the operon in the same orientation (Maseda et al., 2000). The results from DNA sequence analysis revealed that an 8-bp insertion in *mexT* was omitted in all four *nfxC*-type mutants selected. This absence may be a result of additional mutation (s) or deletion (s) in *mexT* that converted the inactive form of MexT to its active form, resulting in overexpression of MexEF-OprN (Maseda et al., 2000). MexT in these clinical isolates harbored an additional mutation Phe(TTC)-129-Ile(ATC) and therefore, it was expected to be inactive. Still, all these four isolates overproduced MexEF-OprN. This observation suggests the existence of uncharacterized regulatory mechanism (s) of MexEF-OprN expression besides MexT. Some studies demonstrated that MexEF-OprN expression was modulated by MexS (Sobel et al., 2005) and MvaT (Westfall et al., 2006). On the contrary, another study suggested the existence of uncharacterized-regulatory mechanism (s) that was not associated with *mexT*, *mexS* or *mvaT* (Wolter et al., 2008). Further studies are warranted to elucidate machinery regulation of MexEF-OprN expression in the clinical isolates in this study.

In the present study, none of the *P. aeruginosa* isolates expressed MexCD-OprJ, in agreement with previous studies in the animal (Beinlich et al., 2001) and human isolates (Llanes et al., 2011; Poonsuk et al., 2014). This confirms that the *P. aeruginosa* isolates producing MexCD-OprJ is scarce in clinical settings for both animals and humans.

In conclusion, the observations highlighted the coexpression of Mex systems that could superimpose their antimicrobial efflux capability and the significance of Mex systems that are normally silent in the *P. aeruginosa* isolates form dogs and cats. For new development in antibiotic therapy, the results suggest that the broad-spectrum EPIs, which are active against all known Mex systems, are more efficient in their potentiating activity of antibiotics and therefore, are more clinically functional and useful.

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บทคัดย่อ

การแสดงออกของระบบ Multidrug efflux 3 ระบบพร้อมกันใน *Pseudomonas aeruginosa* ดื้อยาหลายชนิดพร้อมกันที่แยกได้จากสุนัขและแมว

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การวิจัยครั้งนี้มีวัตถุประสงค์เพื่อทดสอบการแสดงออกของระบบ Mex จำนวน 4 ระบบที่มีความสำคัญทางคลินิก คือ ระบบ MexAB-OprM, MexCD-OprJ, MexEF-OprN และ MexXY ใน *Pseudomonas aeruginosa* ที่แยกได้จากสุนัขและแมว เชื้อเหล่านี้ดื้อยาที่มีความสำคัญทางคลินิกในระดับสูง เมื่อทดสอบด้วย RT-PCR พบว่าเชื้อทุกตัวมีการแสดงออกของระบบ Mex จำนวน 3 ระบบพร้อมกัน คือ MexAB-OprM, MexEF-OprN และ MexXY ไม่พบเชื้อที่มีการแสดงออกของระบบ MexCD-OprJ เมื่อตรวจวัดระดับการแสดงออกของ *mexF* ด้วยเทคนิค quantitative real-time RT-PCR พบว่าระดับการแสดงออกของ *mexF* มีความหลากหลาย (4-219 เท่า) แต่สูงกว่า PAO1 อย่างน้อย 4 เท่า จากการถอดรหัสพันธุกรรมของยีน *mexT* พบว่า ยังมีระบบอื่นๆที่ควบคุมการแสดงออกของระบบ MexEF-OprN ผลการวิจัยชี้ให้เห็นถึงความสำคัญของระบบ Mex ต่อการมีส่วนร่วมในการดื้อยาหลายชนิดพร้อมกันของ *P. aeruginosa* ที่แยกได้จากสุนัขและแมว

คำสำคัญ: แมว สุนัข ระบบ multidrug efflux การดื้อยาหลายชนิดพร้อมกัน สูโดโมนาส แอรูจิโนซ่า

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