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Contribution of the MexXY Multidrug Efflux Pump and Other Chromosomal Mechanisms on Aminoglycoside Resistance in *Pseudomonas aeruginosa* Isolates from Canine and Feline Infections

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ABSTRACT. As study of multidrug efflux pumps is a crucial step for development of efflux pump inhibitors for treatment of *Pseudomonas aeruginosa* infection, the objective of this study was to examine the contribution of the MexXY multidrug efflux systems and other chromosomal mechanisms in aminoglycoside (AMG) resistance in *P. aeruginosa* isolated from dogs and cats. Thirteen *Pseudomonas aeruginosa* isolates from canine and feline infections were examined for contribution of the MexXY multidrug efflux pump and four other chromosomally-encoded genes including PA5471, *galU, nuoG* and *rplY* to AMG resistance. All the isolates were resistant to multiple AMGs and expressed *mexXY*. Deletion of *mexXY* caused 2- to 16-fold reduction in AMG MICs. Overproduction of MexXY did not fully account for the observed AMG resistance. No good correlations were detected between MexXY transcription level and AMG MICs. While no mutations were found in *mexZ,* PA5471 expression varied and its impact on MexXY expression and AMG resistance is diverse. No mutations were found in *galU*. Only two isolates carried a single base change G-367-T in *rplY*. Complete transcription of *nuoG* was detected in all the isolates. In conclusion, the MexXY multidrug efflux pump plays a role in AMG resistance in the dog and cat *P. aeruginosa* isolates, while disruption of *nuoG, rplY* and *galU* did not have a significant impact. These results indicate the existence of uncharacterized AMG-resistance mechanisms.

KEY WORDS: aminoglycoside resistance, *mexXY*, *Pseudomonas aeruginosa*.

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Pseudomonas aeruginosa is infamously known as a common cause of chronic and recurrent infections in both humans and animals, of which the most-notably diseases in dogs and cats include otitis externa/media, urinary tract infection and pyoderma [9, 10, 20]. Treatment of *P. aeruginosa* infection is challenging, because the pathogen intrinsically exhibits and efficiently develops high resistance to several antimicrobials structurally and functionally unrelated, leading to multidrug resistance [1, 24].

As many antipseudomonal drugs are available for therapeutic use in dogs and cats, aminoglycosides (AMGs) are considered a vital component of antipseudomonal chemotherapy [22] due to their efficacy, safety and reasonable price. However, panaminoglycoside resistance e.g., gentamicin, spectinomycin, streptomycin and amikacin has been increasingly reported in the *P. aeruginosa* dog and cat isolates [18, 19, 23]. Such resistance has been clarified to be predominantly due to a poorly understood mechanism namely "impermeability resistance" as a consequence of diminished drug uptake and/or accumulation [21, 31]. The MexXY efflux system, a multidrug efflux pump in the resistance-nodulation-cell-division (RND) family, is involved in the reduced level of AMG accumulation implicated in both impermeability-type and adaptive-type AMG resistance [12, 21, 22]. The MexXY efflux pump is encoded by the *mexXY* operon and evidently serves as the major AMG-resistance mediating system in *P. aeruginosa* clinical isolates. This system additionally confers resistance to nonaminoglycosides, including tetracycline, erythromycin and fluoroquinolones [16]. Inhibition of the MexXY efflux pump appears to be a promising approach for restoring the activity of the existing antimicrobials and improving the *P. aeruginosa* infection [27]. In addition to MexXY, chromosome-encoded genes including *mexZ*, PA5471, *nuoG, rplY* and *galU* have a cumulative contribution to AMG MICs in the *P. aeruginosa* laboratory strain [8].

MexZ is the TetR family transcriptional regulator that binds to the *mexZ-mexX* intergenic region located between positions −104 to −66 bp and represses transcription of the *mexXY* operon [17]. Recently, PA5471-dependent overexpression of MexXY was demonstrated. The PA5471 product modulates transcription of MexXY by binding to MexZ and reduced MexZ DNA-binding ability [34]. The combination amino acid substitution in MexZ and overexpression of PA5471 causes increased *mexXY* expression, leading to AMG resistance. Individual disruption of *nuoG, rplY* and *galU* led to gradual increases in AMG MICs in PAO1. The *nuoG* gene belongs to the *nuoABDEFGHIJKLMN* operon and encodes type I NADH dehydrogenease required for the synthesis of protonmotive force [26]. Inactivation of *nuoG* results in impaired membrane energy, leading to decreased AMG uptake. The *rplY* gene encodes the L25-ribosomal protein that is an AMG-target site, therefore, mutations in

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Table 1. Bacterial strains and plasmid used in this study

Strain		Relevant properties	Source or reference
P. aeruginosa	PAO1	Wild type expressing MexAB-OprM	[29]
	PA049	PAO1Δ(*mexXY*)::*FRT*	[4]
	PA3579	PAO1 with Δ*mexZ*	[31]
	PAO267	PA3579 with Δ(*mexAB-oprM*)	[1]
	PAO280	PA267 with Δ(*mexXY*)	[1]
	Clinical isolates		
	PAJ226 and PAJ245	Pus from wounds in a cat	This study
	PAJ228, PAJ230 and PAJ238	Feline nasal cavity	This study
	PAJ227 and PAJ229	Urine from a cat	This study
	PAJ233 and PAJ239	Pus from wounds in a dog	This study
	PAJ232, PAJ234 and PAJ240	Canine otitic ears	This study
	PAJ235	Urine from a dog	This study
	Δ(*mexXY*) mutants		
	PAJ262	PAJ226Δ(*mexXY*)::*FRT*	This study
	PAJ264	PAJ227Δ(*mexXY*)::*FRT*	This study
	PAJ266	PAJ229Δ(*mexXY*)::*FRT*	This study
	PAJ268	PAJ230Δ(*mexXY*)::*FRT*	This study
	PAJ270	PAJ232Δ(*mexXY*)::*FRT*	This study
	PAJ272	PAJ234Δ(*mexXY*)::*FRT*	This study
	PAJ274	PAJ239Δ(*mexXY*)::*FRT*	This study
	PAJ277	PAJ240Δ(*mexXY*)::Gm^r-*FRT*	This study
	PAJ276	PAJ245Δ(*mexXY*)::*FRT*	This study
E. coli	SM10	Donor for biparental mating *thi-1 thr leu tonA lacy supE recA::*RP4-2-Tc::*Mu* (Km^r)	[6]
Plasmid	pUCP20	Ap^r; a cloning vector	[30]
	pPS1221	Ap^r, Gm^r: pEX18Ap containing Δ(*mexXY*) ::FRT-*Gm^r* fragment	[2] [31]
	pAMR1	Ap^r, pUCP20 carrying *mexXY*	

Abbreviations: Ap^r, ampicillin resistance; Gm^r, gentamycin resistance, Km^r, kanamycin resistance.

this region contribute to resistance to AMGs [8]. The *galU* gene encodes UDP-glucose pyrophosphorylase essential for the synthesis of LPS-outer core. The disrupted *galU* gene leads to the production of the A and B band-deficient LPS that adversely affects AMG binding and impairs their outer membrane uptake [7]. However, it was recently shown that inactivation of *nuoG, rplY* and *galU* did not play an important role in AMG resistance in the *P. aeruginosa* cystic fibrosis (CF) isolates [14].

Up to date, efflux pump inhibitors for combating antimicrobial resistance caused by multidrug efflux pumps have been researched only for medical treatment in humans [27]. These molecules could be discovered and/or developed as a novel strategy for treatment of *P. aeruginosa* infection in dogs and cats. In this case, study of contribution and functions of multidrug efflux pumps in antimicrobial resistance is a crucial step to achieve the new therapeutic goal. However, role of MexXY in AMG resistance has been systematically studied in *P. aeruginosa* from CF patients [13, 28, 33]. In contrast, such information is still limited in the *P. aeruginosa* veterinary isolates. We previously examined contribution of the MexXY efflux pump in AMG resistance in the isolates from cow mastitis [4]. However, there have been no published data on the MexXY efflux pump of the *P. aeruginosa* isolates from dogs, and cats, and different types of antimicrobials are used in food-producing, and companion animals. Therefore, the aim of this study was to investigate the involvement of MexXY in AMG resistance in the *P. aeruginosa* clinical isolates from dogs and cats. Additional-chromosomal mechanisms including PA5471, *nuoG, rplY* and *galU* were also examined.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions: All the bacterial strains and plasmids used in this study are shown in Table 1. Thirteen *P. aeruginosa* clinical isolates were obtained from the strain collection of the Veterinary Diagnostic Laboratory (VDL), Faculty of Veterinary Science, Chulalongkorn University. They 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. These dogs and cats were from different households.

Isolation of *P. aeruginosa* was performed using standard methods [15]. Briefly, the bacteria were isolated on blood agar. The colonies with hemolytic zone and pyocyanin-nonfluorescent bluish pigment were collected and further confirmed to be *P. aeruginosa* by using biochemical tests. Only a single *P. aeruginosa* colony was taken from each positive sample at only one time. The bacterial isolates were grown either on Luria Bertani (LB) agar (Difco, BD Diagnostic Systems, Sparks, MD, U.S.A.), in LB broth (Difco), in Mueller-Hinton broth (MHB; Difco) with adjusted concentrations of Ca^{2+} and Mg^{2+} and on Mueller-Hinton agar (MHA; Difco). Antibiotics used in selective medium were as follows: ampicillin, 100–150 μg/m*l* and gentamicin, 15 μg/m*l* for *Escherichia coli* and carbenicillin, 200 μg/m*l* and

Table 2. Primers used in this study

Gene/region	Primer	Sequence (5'–3')	Reference
Mutation			
mexZ -mexZX intergenic region	mexZXup	CGCAGAATTCCGGCGTCCGC	[4]
	mexZXdown	GCAAGCTTCTGCACATCAGCGAG	
rplY	rplYF144-U	ATCGCCCGAACGCTGGT	[14]
	rplYF144-L	ATGCCGGGTCTGGTCGTATTC	
galU	galUF14o-U	CGAGCGCAGCCTGATTAGACT	[14]
	galUR1121-L	ACAGCTCAGGTAGGGCGGATA	
RT-PCR			
mexY a)	mexYRTUP	AGCTACAACATCCCCTA	[4]
	mexYRTdown	AGCACGTTGATCGAGAAG	
nuoH	nuoHF657-U	GCAGGAACTGGCGGACGG	[14]
	nuoHL823-L	GGTCTTGGCGGCGAAGTAGAA	
qRT-PCR			
PA5471	PA5471-U	CGACATCGGCTGTGGCA	[14]
	PA5471-L	AGTCGCTCCAGGTCTCGTC	
rpsl	rpsLrealtimeup	CGGCACTGCGTAAGGTATG	[4]
	rpsLrealtimedown	CCCGGAAGGTCCTTTACACG	
AMG-Modifying enzymes			
aadA1	aadA1-F	CTCCGCAGTGGATGGCGG	[3]
	aadA1-R	GATCTGCGCGCGAGGCCA	
aadA2	aadA2-F	CATTGAGCGCCATCTGGAAT	[3]
	aadA2-R	ACATTTCGCTCATCGCCGGC	
aadB	aadB-F	CTAGCTGCGGCAGATGAGC	[3]
	aadB-R	CTCAGCCGCCTCTGGGCA	
aac(3')-Ia	aac3Iaup	CTGACCAAGTCAAATCCATGCGGG	[4]
	aac3Iadown	CCACTGCGGGATCGTCACCG	
aac(6')-IIa	aac6IIaup	AGAGCGATGGCGGAAGAGTCC	[4]
	aac6IIAdown	ATCCTGCCTTCTCATTGCAGCG	
aac(6')-IIb	aac6IIbup	CCGAAGAAGGAGTGACGCCG	[4]
	aac6IIbdown	GCGCAAACCGTTCACCAACGG	
aph(3')-IIb	aph3IIbup	GAACGAAACCCAGAGCGACGG	[4]
	aph3IIbdown	CAATCGATGAAGCCGCTGAAGC	
ant(2'')-Ia	ant2Iaup	TGGAGCAGCAACGATGTTACGC	[4]
	ant2Iadown	CCACTGGTGGTACTTCATCGG	
strA	strA-F	TGGCAGGAGGAACAGGAGG	[3]
	strA-R	AGGTCGATCAGACCCGTGC	
strB	strB-F	GCGGACACCTTTTCCAGCCT	[3]
	strB-R	TCCGCCATCTGTGCAATGCG	

a) Also used for qRT-PCR of *mexY*.

gentamicin, 50 μg/m*l* for *P. aeruginosa*. *E. coli* SM10 was a donor in conjugation experiment.

Antimicrobial susceptibility testing: Antimicrobial susceptibilities were examined by determination of minimum inhibitory concentrations (MICs) using the standard two-fold microdilution technique according to Clinical and Laboratory Standards Institute guidelines (CLSI) [5]. The MIC breakpoints used in this study were from CLSI, when available (Table 3). However, the specific-CLSI breakpoints for *P. aeruginosa* are not available for all antimicrobials (i.e., streptomycin, neomycin and tobramycin). Therefore, the CLSI interpretive breakpoints for the Enterobacteriaceae and those in the published data were used for the antimicrobials lacking the CLSI breakpoints [23]. Experiments were performed in triplicates and repeated independently twice. *P. aeruginosa* ATCC 27853 and wildtype PAO1 were used as quality control organisms.

General DNA techniques: Chromosomal DNA was extracted and purified using the QIAamp mini kit (Qiagen, Hilden, Germany). Plasmid DNA was prepared using QIAprep® Mini-spin kit (Qiagen). Selected DNA fragments were purified from agarose gels using QIAquick Gel Extraction kit (Qiagen).

Construction of unmarked chromosomal Δ(mexXY) mutants: pPS1221 was used as a source for Δ(*mexXY*) mutant alleles [2] to delete chromosomal *mexXY* operon by using Flp/*FRT* recombinase-mediated excision technology as previously described [1, 11]. Gene replacement at the *mexXY* operon was confirmed by PCR and DNA sequencing analyses. Resistance phenotypes of the parents and their corresponding Δ(*mexXY*) mutant derivatives were confirmed by complementation analyses using pAMR-1 [31].

PCR, reverse transcription (RT)-PCR and DNA sequencing: PCR amplifications were performed using KAPATaq ReadyMix DNA polymerase (Kapabiosystems, Boston, MA, U.S.A.) by following the manufacturer's protocol. All the primer pairs used for PCR amplification are listed in Table 2. The DNA fragments encompassing *mexZ* and the *mexZ-mexX* intergenic region, *rplY* and *galU* were PCR-amplified from genomic DNA template. All the AMG-modifying en-

Table 3. MICs, transcription level of *mexY* and PA5471 and mutations in the *P. aeruginosa* clinical isolates and their corresponding Δ*mexXY* derivatives

Strains	MIC(breakpoint) (μg/ml) [a]							Transcription level		Mutations		AMG-modifying enzyme
	AMK(64)	GEN(8)	KAN(64)	NEO(10)	STR(64)	SPC(128)	TOB(8)	*mexY*	PA5471	*mexZ-mexX* [b,c]	*rplY*	
PAO1	**8**	**1.6**	64	**16**	16	512	1	1	1	–	–	–
PA049	**2**	**0.2**	32	**4**	8	512	0.5					–
PAJ226	**8**	**2**	**256**	32	**32**	**2,048**	**8**	24	6	A(-112)-G	G-367-T(Ala-123-Ser)	*aadB*, *aac(3')-Ia*, *strAB*
PAJ262	**0.5**	**<0.25**	**32**	16	**4**	**128**	**1**					ND
PAJ227	**2**	**4**	**8**	**4**	**64**	**2,048**	**4**	191	16	–	–	–
PAJ264	**0.25**	**1**	**2**	**0.5**	**16**	**256**	**0.5**					–
PAJ229	**8**	**4**	**256**	**128**	**128**	**1,024**	**2**	4.7	1	–	–	*aadB*, *aac(3')-Ia*
PAJ266	**1**	**0.5**	**64**	**8**	**8**	**128**	**0.5**					ND
PAJ230	**8**	**4**	**128**	**16**	**64**	**1,024**	**2**	26	16	–	–	*aadA2*, *aac(3')-Ia*
PAJ268	**1**	**0.5**	**32**	**4**	**4**	**256**	**0.25**					ND
PAJ232	4	**4**	128	16	32	**1,024**	**128**	0.03	28	–	–	*aadA1*, *aadB*, *aac(3')-Ia*
PAJ270	2	**1**	64	8	32	**256**	**16**					ND
PAJ234	**8**	**8**	128	**128**	**512**	**2,048**	2	6	13	–	–	*aadB*, *aac(6')-IIa*
PAJ272	**1**	**1**	64	**16**	**128**	**256**	1					ND
PAJ239	**8**	**2**	128	**8**	**128**	**1,024**	1	21	2	–	–	–
PAJ274	**2**	**0.5**	64	**0.5**	**8**	**256**	0.5					–
PAJ240	16	8	**256**	**128**	128	**2,048**	**4**	3	1	A(-112)-G	–	–
PAJ277	2	ND	**64**	**32**	64	**512**	**1**					–
PAJ245	**4**	**4**	**256**	**32**	**64**	**2,048**	0.5	6	2	–	G-367-T(Ala-123-Ser)	*aadA2*, *aac(3')-Ia*
PAJ276	**0.5**	**<0.25**	**64**	**8**	**8**	**128**	0.25					ND
PAJ228 [d]	4	256	128	8	512	1,024	256	6	12	C(-11)-G, G(-57)-A, A(-88)-T, G(-99)-T, G(-106)-C, A(-112)-G	–	*aadB, aadA1, aadA2*, *aac(3')-Ia*, *strAB*
PAJ233 [d]	8	8	128	32	64	1,024	1	1	86	–	–	*aadB*, *aac(3')-Ia*
PAJ235 [d]	16	128	256	16	512	1,024	4	8	19	G(-57)-A, A(-112)-G	–	*aadA2*, *strAB*
PAJ238 [d]	8	4	128	32	512	1,024	1	21	1	A(-112)-G	–	–

Abbreviations and concentration ranges (in parenthesis): AMK, amikacin (1–2,048 μg/ml); GEN, gentamicin (1–256 μg/ml); KAN, kanamycin (1–2,048 μg/ml); NEO, neomycin (1–256 μg/ml); STR, streptomycin (1–2,048 μg/ml); SPC, spectinomycin (1–2,048 μg/ml); TOB, tobramycin (1–128 μg/ml). ND, not detected. a) Values in boldface indicate MICs for the parent strains that are at least 4-fold different from those for the corresponding Δ*mexXY* derivatives. b) *mexZ-mexX* intergenic region. c) Negative number indicates the positions of base pair changes relative to the first nucleotide of the *mexX* ATG start codon. d) Deletion of the *mexXY* operon was not successful.

zyme encoding genes were amplified using whole cell DNA templates.

Reverse transcription (RT)-PCR was performed to detect transcription of *mexY* and *nuoH*. Total RNA isolation of *P. aeruginosa* was carried out using Qiagen RNeasy Mini kit (Qiagen) and treated with RNase-free DNaseI (Invitrogen, Carlsbad, CA, U.S.A.). One-μg DNase treated RNA was reversed transcribed to single stranded-cDNA using ImProm-II™ Reverse Transcriptase (Promega, Madison, WI, U.S.A.) with the reverse primers specific to *mexY* (i.e. mexYRTdown) and *nuoH* (i.e.nuoHL823-L) and then, the cDNA obtained was used as the template for PCR amplification using the specific primer pairs as described above.

For nucleotide sequencing analyses, the PCR products were purified using the QIAQuick Gel Extraction kit (Qiagen) and submitted for sequencing at Macrogen Inc. (Seoul, South Korea) using the PCR primers. The nucleotide sequence of each purified PCR product was determined on both strands, and the DNA-sequencing results were compared with PA2023 and PA2020 available at the Pseudomonas Genome Project (http://pseudomonas.com) for *galU* and *mexZ*, respectively [32].

Quantitative real-time PCR (qRT-PCR): Transcription level of the *mexY* and PA5471 genes was assessed by qRT-PCR as previously described with some modifications [4, 14]. The cDNA was synthesized from 1 μg of total RNA as described above and quantified using KAPA SYBR® FAST qPCR kit (Kapabiosystems). PCR assays were done in triplicates for all the genes. The Ct values from two separate experiments (SD<0.1) were used to estimate the average cDNA copy numbers for each sample, and the *rpsL* gene was used as internal control. The average *mexY* and PA5471 cDNA copy number was normalized with that of of *rpsL*, and the transcription levels were identified as fold change-ratios to that of the reference strain PAO1.

RESULTS

Aminoglycoside susceptibility in P. aeruginosa isolates from canine and feline infections: All of the *P. aeruginosa* isolates were examined for their AMG susceptibility (Table 3). All exhibited resistance to spectinomycin and most isolates showed intermediate resistance to streptomycin, kanamycin and neomycin. Only few isolates were resistant to gentamicin (5 isolates) and tobramycin (3 isolates). None of the isolates were resistant to amikacin.

Effects of mexXY loss on AMG resistance: To further assess the participation of MexXY in AMG resistance in all isolates, the *mexXY* operon was deleted to generate unmarked Δ(*mexXY*)::*FRT* mutants and the impact on AMG resistance was evaluated. However, construction of unmarked Δ(*mexXY*)::FRT mutants was completed in only eight isolates. In addition, the Gm^r-*FRT* cassette was not successfully excised in one isolate (i.e., PAJ240), resulting in the Δ(*mexXY*):: Gm^r-*FRT* marked mutant (i.e., PAJ277) where the association of MexXY to gentamicin resistance was not assessed.

The effect of MexXY loss on AMG resistance varied among the isolates in this collection. Deletion of *mexXY* resulted in 2- to 16 fold decline in AMG MICs in all nine isolates. Constitutive expression of MexXY from pAMR-1 fully restored the AMG susceptibilities, confirming the influence of Δ(*mexXY*) on all the AMG MICs observed (data not shown). The loss of *mexXY* reduced the MICs of all AMGs (4- to 16-fold) in three isolates (i.e., PAJ227, PAJ229 and PAJ230) and six of the seven AMGs in two isolates (i.e., PAJ226 and PAJ245). The absence of MexXY had only moderate effects (two- to fourfold decline in MIC) on AMG resistance in many strains. For example, the MICs for all AMGs tested of PAJ277 were only two- to fourfold reduced from those of the parents PAJ240. When consider individual AMG substrates, consequences of *mexXY* loss were different for the same substrate in different *P. aeruginosa* host strain. For instance, *mexXY* loss generated only a marginal effect on streptomycin susceptibility in two isolates (PAJ232 and PAJ240), but resulted in 16-fold decreased streptomycin MIC in three isolates (i.e., PAJ229, PAJ230 and PAJ239).

Quantitation of mexXY and PA5471: To evaluate the association between *mexXY* expression and AMG resistance, *mexXY* expression was initially measured by RT-PCR and their transcription was observed in all clinical isolates (data not shown). Then, the relative transcription level of *mexXY* was assessed. The Ct values of the *rpsL* internal control from different isolates were comparable with each other in all qRT-PCR experiments. The *mexXY* operon was found to overproduce three to 191-fold higher than PAO1 in 11 isolates. A decreased transcription of *mexXY* was detected in one isolate (i.e., PAJ232, 0.03 fold less than PAO1). The last isolate i.e., PAJ233 produced MexXY at the level equivalent to that of PAO1.

Relative expression of PA5471 was measured by qRT-PCR in all isolates. Of all the isolates tested, PA5471 was upregulated in 10 isolates, and its transcription level varied from 2- to 86-fold. The variability of PA5471 transcription level was observed among the isolates with comparable MexXY expression. For instance, PAJ234, PAJ228 and PAJ245 that expressed 6-fold MexXY produced PA5471 2- to 13-fold. Similarly, the strains with the similar PA5471 transcription level (i.e., PAJ227, PAJ230 and PAJ239) did not express MexXY at the same level. The strains with higher PA5471 expression produced neither higher *mexXY* transcriptional level nor higher AMG MICs than those with less PA5471 expression. This is best illustrated with PAJ233 that was not more resistant to AMGs than PAJ288 and PAJ235.

Sequencing analysis of mexZ, rplY and galU: The involvement of *mexZ* and the *mexZ-mexX* intergenic region in *mexXY* expression was examined by DNA sequencing in all isolates. The *mexZ* sequences in all the *P. aeruginosa* isolates tested were homologous to that of PAO1, while five isolates (i.e., PAJ226, PAJ240, PAJ228, PAJ235 and PAJ238) contained nucleotide changes in the *mexZ-mexX* intergenic region. A replacement of A at position −112 (relative to position 1 being the A of the *mexX* start codon) with G was most commonly found. Expression of MexXY in three isolates carrying only A (−112)G varied from three to 24-fold compared to PAO1. One isolate contained up to 6 single

base mutations (i.e., PAJ228), and its MexXY transcription level was comparable to that of the isolates carrying two single base mutations (i.e., PAJ235). The PAJ227 strain with highest MexXY transcription level contained no mutation within the *mexZ-mexX* intergenic region. Some isolates with the *mexZ-mexX* intergenic region indistinguishable from that of PAO1 (i.e., PAJ234 and PAJ245) had MexXY expression comparable to that of the strains carrying mutations in the region (i.e., PAJ228 and PAJ235).

The *galU* and *rplY* genes were sequenced in all isolates. The resulting DNA sequences showed that the nucleotide sequence of *galU* in all isolates was identical to that of PAO1. Only two isolates (i.e., PAJ226 and PAJ245) contained a G-367-T single point mutation in *rplY*, leading to an Ala-123-Ser amino acid substitution in RplY.

Expression of nuoG: Transcription of *nuoH* located downstream of *nuoG* was investigated by using RT-PCR in all isolates. Expression of *nuoH* was observed in all the isolates.

The presence of AMG-modifying enzyme encoding genes: Most isolates except PA227, PA238, PA239 and PA240 were positive to AMG-resistance genes tested (Table 3). Some strains carried several genes encoding resistance to an individual AMG e.g., PAJ228 and PAJ237. The genes most commonly identified were *aadB*(7 isolates) and *aac(3')-Ia* (7 isolates). None of the isolates were found to contain *aph(3')-IIb, ant(2'')-Ia* and *aac(6')-IIb.*

DISCUSSION

In the present study, the *P. aeruginosa* isolates from canine and feline infections were resistant to panaminoglycosides. High resistance rates to spectinomycin, streptomycin, kanamycin and neomycin were observed, in agreement with a previous study [20]. This may be not surprising, since these antibiotics are commonly prescribed in dogs and cats due to their relatively-less expensive cost. In contrast, lack of amikacin resistance and low resistance rates to gentamicin and tobramycin were observed. Both gentamicin and amikacin are antibiotics of last resort due to their common side effects of kidney damage and hearing loss. Similarly, tobramycin produces side effects and is not used long-term. This limited usage could be an explanation for such low resistance rates observed.

Diverse contribution of MexXY to AMG resistance among the dog and cat isolates in this study was similar to that previously reported in the *P. aeruginosa* veterinary isolates [4]. The loss of *mexXY* caused up to 4- to 16-fold reduction in MICs of several AMGs in many isolates, suggesting the significant contribution of the MexXY efflux pump in panaminoglycoside resistance. The moderate effects of *mexXY* loss on AMG resistance in several strains suggested the existence of other AMG-resistance determinants that were not characterized in this study. Such marginal effect of *mexXY* loss on resistance to clinically-important AMGs was previously observed in the cow isolates [4] and could create clinical impact by preventing AMGs from reaching their optimal concentrations, especially where the antibiotic penetration is impeded. Impact of MexXY loss on a single AMG was different in different *P. aeruginosa* host strains. This is mostly due to the different combination of AMG-resistance mechanisms additionally expressed and compromised *mexXY* loss in each individual strain.

In addition, most isolates harbored AMG-modifying enzyme encoding genes confirming the existence of additional AMG-resistance mechanisms in the *P. aeruginosa* strains. However, the contribution level of these genes to AMG resistance is still unclear. Further investigations are required to disclose their significance level.

Expression of *mexXY* and its association with AMG resistance varied. The strains with the highest *mexXY* expression (i.e., PAJ227) did not show greater resistance to AMGs than those with much less *mexXY* expression (i.e., PAJ234, PAJ240, PAJ228, and PAJ235). The strain with a decreased transcription of *mexXY* was resistant up to four AMGs. Taken together, the data supported that *mexY* transcription was not associated with AMG resistance. This phenomenon was previously observed in the animal and human isolates [4, 25]. Therefore, high *mexXY* expression level is not an absolute marker for high AMG resistance level among the *P. aeruginosa* clinical isolates.

The predominant nucleotide change on MexZ, A(−112) G, was located outside of the MexZ-binding domain, but still situated in the predicted promoter of *mexX* located between positions −88 and −133 [4]. It was found that the presence and the number of single base mutations were not correlated to the MexXY transcription level, and the absence of mutation within the *mexZ-mexX* intergenic region did not guarantee the low MexXY transcription level. Based on the observations, it is unlikely that the mutations in the *mexZ-mexX* intergenic region have substantial impacts on MexZ functioning as a *mexXY* repressor.

The *P. aeruginosa* strains with comparable MexXY transcription level expressed PA5471 at different level (2- to 13 fold), and MexXY transcription level among the strains with comparable PA5471 transcription level was quite different. Therefore, there was a lack of good correlation between PA5471 expression level and *mexXY* transcription level. Two isolates i.e., PAJ233 and PAJ232 overexpressed PA5471 up to 86 and 28 fold, respectively, but did not overproduce MexXY, in agreement with a previous study in the CF isolates [14]. In *vice versa*, three isolates with PA5471 expression level comparable to that of PAO1 (i.e. PAJ229, PAJ240 and PAJ238) had elevated-MexXY expression (3- to 21-fold). These results suggest the existence of additional regulatory mechanisms that manipulate *mexXY* expression in the isolates. The strains with higher PA5471 expression produced neither higher *mexXY* transcriptional level nor higher AMG MICs than those with less PA5471 expression. This is best illustrated with PAJ233 that was not more resistant to AMGs than PAJ288 and PAJ235. Hence, the prominent PA5471 expression cannot be a conclusive indicator of high *mexXY* expression and high AMG resistance.

Sequencing analyses revealed the presence of single point mutation, A G-367-T, only in *rplY* from two isolates. This nucleotide change may have an impact on AMG resistance; however, its actual effect on AMG MICs was not investi-

gated in this study. Due to its location downstream of *nuoG*, expression of *nuoH* was examined to confirm the complete transcription of *nuoG*. Expression of *nuoH* was observed in all the isolates, indicating the *nuoG*-complete transcription and suggesting that the alteration of this gene did not play a role in AMG resistance among the veterinary strains in this study. Altogether, changes of *nuoG, rplY* and *galU* did not significantly contribute to AMG resistance level in the dog and cat isolates, similar to a previous observation in the human clinical isolates [14].

In conclusions, the results obtained in this study showed the contribution of the MexXY efflux pump in AMG resistance in the *P. aeruginosa* isolates from canine and feline infections. MexXY expression alone cannot explain the whole AMG resistance observed. The presence of MexXY-regulatory mechanisms additional to *mexZ* and PA5471 is suggested. The effects of *nuoG*, *rplY* and *galU* on AMG resistance were insignificant in AMG resistance of the *P. aeruginosa* dog and cat isolates. Further studies are warranted to elucidate the whole picture of MexXY-regulatory machinery in the AMG-resistant mutants in this study. Furthermore, the systematic surveillance of antimicrobial resistance that is usually performed in the pathogens from humans and livestocks is required for those from companion animals. The prudent guideline for antimicrobial use should be elaborated for pet animals to prevent and control the emergence and dissemination of the multidrug-resistant *P. aeruginosa* strains.

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