Molecular mechanisms of ceftazidime resistance in *Pseudomonas aeruginosa* isolates from canine and human infections

S.-J. Du[1], H.-C. Kuo[2], C.-H. Cheng[1], A.C.Y. Fei[1], H.-W. Wei[3], S.-K. Chang[1]

[1]Graduate Institute of Veterinary Medicine, National Taiwan University, Taiwan, ROC
[2]Department of Veterinary Medicine, National Chiayi University, Chiayi, Taiwan, ROC
[3]Department of Animal Science and Technology, National Taiwan University, Taipei, Taiwan, ROC

ABSTRACT: Sixty-six clinical *P. aeruginosa* isolates, 17 obtained from canine otitis specimens and 49 received from human patients with bloodstream infections, were collected between February 2007 and January 2008. The minimal inhibitory concentrations (MICs) of the antimicrobial agents of these isolates were determined. Multidrug resistance was common, with 23 (34.8%) isolates found to be ceftazidime resistant. To explore the mechanisms of ceftazidime resistance, PCR analyses were performed to detect drug-resistance genes. The prevalence rate of Ambler class A, B, and D β-lactamase genes were obtained, with $bla_{\text{TEM-1}}$ 100%, $bla_{\text{PSE-1}}$ 100%, $bla_{\text{OXA-2}}$ 96.2%, $bla_{\text{SHV-18}}$ 91.3%, $bla_{\text{OXA-17}}$ 78.3%, $bla_{\text{VIM-3}}$ 26.1%, $bla_{\text{OXA-10}}$ 21.7% and $bla_{\text{SHV-1}}$ 8.7%. An efflux inhibition assay with the PAβN compound was conducted. The ceftazidime resistance isolates were also tested by RT-qPCR to determine the mRNA expression levels of the *oprM* and *ampC* genes. Five (21.7%) of the ceftazidime resistance isolates appeared to overactivate the OprM efflux system. The *ampD*, *ampE*, and *ampR* genes and the *ampC-ampR* intergenic region were subsequently amplified and sequenced. Five (21.7%) of the ceftazidime resistance isolates from humans and canines had a point mutation in AmpR (Asp135-Asn, n = 3; Als194-Ser, n = 2), which induces AmpC overproduction from 10- to 80-fold. This study first reported ceftazidime resistance in *P. aeruginosa* from canine otitis specimens, which are closely related to ESBLs (50%), including the overproduction of AmpC (25%) and the OprM efflux system (25%). The ESBLs (100%) played an important role in all ceftazidime resistance isolates from humans, and either AmpC (21.1%) or OprM (21.1%) might be overexpressed within the same isolate. A human patient isolate (H307B) showed simultaneous expression of ESBLs, the OprM efflux system, and AmpC overproduction.

Keywords: *ampC*; *ampR*; extended spectrum β-lactamases; OprM efflux system

Pseudomonas aeruginosa causes septicemia and nosocomial infections in humans (El Amary et al., 2001; Berthelot et al., 2005) and otitis externa, pyoderma, wound infections and abscesses in canines (Griffin, 1993; Cole et al., 1998; Scott et al., 2001; Peterson et al., 2002). Ceftazidime is an important and effective antimicrobial agent for the therapy of serious infections due to multidrug resistance in *P. aeruginosa*. A surge in ceftazidime resistance in human clinical isolates of *P. aeruginosa* results from the production of acquired β-lactamase, the constitutive overproduction of AmpC, or an activation of the MexAB-OprM or MexXY-OprM efflux systems (Lindberg et al., 1987; Li et al., 1994; Stapleton et al., 1995; Nordmann and Guibert, 1998; Aires et al., 1999; Kuga et al., 2000; Masuda et al., 2000; Livermore, 2002). The molecular mechanism of canine ceftazidime resistance in *P. aeruginosa* isolates still requires further clarification. In this study, we attempt to elucidate the different ceftazidime resistance mechanisms between canine and human isolates of *P. aeruginosa*.

MATERIAL AND METHODS

Bacterial isolates and antimicrobial susceptibility test

This study included 66 isolates identified as *P. aeruginosa* by API 20NE (bioMérieux, Marcy l'Etoile, France). Seventeen were collected from canine otitis specimens in a veterinary teaching hospital, and 49 were collected from human patients with bloodstream infections in a number of hospital microbiology laboratories, between February 2007 and January 2008. Antimicrobial susceptibility tests followed the broth microdilution method of performance standards for antimicrobial susceptibility testing (CLSI, 2006). The antimicrobial agents used were ciprofloxacin and enrofloxacin (Fluka Chemie, Buchs, Switzerland); ampicillin, aztreonam, ceftazidime, chloramphenicol, colistin, and gentamicin (Sigma Aldrich, St Louis, MO, USA); cefepime (Bristol-Myers Squibb, Hounslow, UK); and meropenem (Astra-Zeneca, Macclesfield, UK). Reference standards of *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as controls on each plate to determine minimal inhibitory concentrations (MICs). All bacteria were incubated in Mueller Hinton broth with the aforementioned antimicrobial agents under a variety of concentrations at 37°C for 16–18 hours. Bacterial growth was subsequently inspected via observation of turbidity.

Amplification and sequencing of β-lactamase genes

The detection of bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{PER}, bla_{VIM}, bla_{IMP}, bla_{SPM}, bla_{VEB}, bla_{GES} and bla_{PSE} type extended spectrum β-lactamases (ESBLs) in the ceftazidime resistance isolates was performed by PCR with the specific primers listed in Table 1 and under conditions described elsewhere (Gales et al., 2003; Weldhagen, 2004; Jiang et al., 2006; Strateva et al., 2007). The total DNA of *P. aeruginosa* isolates was extracted using the InstaGene DNA Purification Matrix kit (Bio-Rad, CA, USA), as recommended by Bio-Rad. The target extracted DNA was amplified by the GeneAmp PCR system 9600 thermal cycler (Applied Biosystems, Foster City, CA). PCR products were cloned into cloning vector pCRII (Invitrogen Life Technologies, Paisley, UK) for DNA sequencing if unexpected sizes of the PCR products were found. All acquired DNA sequences were subjected to the method of Altschul et al. (1997) for comparison with the BLAST online search engine from GenBank at the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov/blast.

Plasmid profile, plasmid DNA restriction length polymorphism, and Southern blot hybridization

To detect the prevalence rate of the bla_{SHV-18} gene, plasmid DNAs were prepared from the 23 ceftazidime resistance *P. aeruginosa* isolates. Plasmid DNA was extracted using the Qiagen Midi Kit (Qiagen, Courtaboeuf, France), digested with the *Pst*I restriction enzyme (TaKaRa, Japan) and then analyzed in a 0.8% agarose gel. Southern blot hybridization was performed according to Sambrook et al. (1989), in which digested plasmid DNA was transferred onto a Hybond N^+ nylone membrane (Nycomed Amersham plc, Buckinghamshire, UK) with a vacuum blotting system. The bla_{SHV-18}-specific probe from the PCR-generated amplicons of bla_{SHV-18} containing *Klebsiella pneumonia* ATCC 700603 was labeled with a PCR DIG-labeling kit (Roche Diagnostics GmbH, Mannheim, Germany). The membrane was pre-hybridized at 42°C for 1 h, and hybridization took place at 42°C for 24 h to denature the bla_{SHV-18} probe. Subsequently, the DIG-High Prime DNA labeling and detection system (Digoxigenin Labeling and Detection Kit; Roche Diagnostics, Mannheim, Germany) was used for signal detection.

Amplification and sequencing genes of the *ampD, ampE and ampR*, and *ampR-ampC* intergenic regions

To determine the presence of *ampD* and *ampE* in each isolate, the PCR procedures and conditions used were as follows: all amplifications were performed in 50 µl reactions containing DNA (2.5 µl), primers (50 pmol each), deoxynucleoside triphosphates (200µM), *Taq* DNA polymerase (1.25 U; Takara Biomedicals, Tokyo, Japan) and 5 µl of 10 × *Taq* polymerase buffer (500mM KCl, 100mM Tris-HCl [pH 8.3], 15mM $MgCl_2$, 0.1% gelatin). Cycling conditions included denaturation for 10 min at 95°C, amplification for 30 cycles of 30 s at 95°C, 1 min at 60°C for *ampD* and 57°C for *ampE*, and 1 min at 72°C, and extension for 10 min at 72°C.

Amplification of *ampR*, including the *ampR-ampC* intergenic region in each isolate, was performed under the following PCR conditions: denaturation for 10 min at 94°C; amplification for 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and extension for 10 min at 72°C. A 20 µl aliquot of the PCR product was electrophoresed in a 1.0% agarose gel and stained with ethidium bromide (50 mg/l). Band sizes of 0.9 kb and 1.2 kb were defined as the *ampD* gene and the *ampE* genes. A size of 1.0 kb was defined as *ampR* including the *ampR-ampC* intergenic region. The remaining PCR products were purified using a PCR purification kit (Qiagen, Valencia, CA, USA) and were sequenced with PCR forward and reverse primers using the ABI PRISM Big Dye cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3700 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The primers used are listed in Table 1. DNA sequence data were analyzed as recommended by GenBank DNA sequences using the National Center for Biotechnology Information via the BLAST network service (Altschul et al., 1997), and were subsequently compared with wild-type PAO1 sequences in GenBank, accession numbers X67095 (*ampR*), X54719 (*ampR-ampC* intergenic region), and AF082575 (*ampD*, *ampE*). The Clustal W method in the Multiple Sequence Alignment Program was used to align and compare the sequences.

RNA isolation

All ceftazidime-resistant *P. aeruginosa* isolates (n = 23) were incubated aerobically in LB (Luria Bertani) broth until mid-log phase. RNA was subsequently isolated with TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). Eight micrograms of RNA from each isolate was treated for 1 hour with 8 units of RO1 RNase-free DNase (Promega, Madison, WI, USA) to eliminate DNA. The SuperScript III first-strand synthesis system (Invitrogen Life Technologies, Paisley, UK) was used to synthesize cDNA from total RNA, and random hexamers were used for RT-PCR.

Detection of *ampC* and *oprM* genes by real-time qPCR

The specific primers listed in Table 1 were used to amplify *ampC* and *oprM* genes in the iCycler iQ5 real-time detection system (Bio-Rad Laboratories) using Sybr Green iQ™ supermix (Bio-Rad Laboratories). Thermal cycling conditions were as follows: 3 min at 95°C, 40 cycles of 10 s at 95°C, and 30 s at 58°C. Melting curves were then analyzed under the following conditions: the temperature was ramped to 95°C for 1 min, cooled to 58°C for 1 min at a slope of 20°C per second, followed by a 0.1°C per second rise in temperature up to 95°C. Triplicate PCR reactions were performed for each isolate. The *rpoD* housekeeping gene was used as an internal standard (Savli et al., 2003), amplified in separate quantity PCR reactions to correct for differences in the mRNA recovery rate. The relative expression of *ampC* and *oprM* was assessed by the $\Delta\Delta C_T$ method as described previously (Livak and Schmittgen., 2001). *P. aeruginosa* ATCC 27853 was used as a control to calculate the ratio of *ampC* and *oprM* gene expression in three experiments for each isolate. To ensure the specific amplification acquired, 1% agarose gel electrophoresis and ethidium bromide staining was carried out to confirm the absence of additional PCR products.

Efflux pump inhibition test

All ceftazidime resistant *P. aeruginosa* isolates (n = 23) were tested for efflux pump inhibition. The ceftazidime MICs were determined by broth microdilution in the absence or presence of 50 mg/l PAβN (Sigma Aldrich, St Louis, MO, USA). The ceftazidime MICs decreased at least 4-fold in the presence of PAβN, likely due to overproduction of the efflux pumps as shown by the positive reaction in the efflux pump inhibition test (Mesaros et al., 2007).

RESULTS AND DISCUSSION

All *P. aeruginosa* isolates from canine and human infections showed 100% resistance to ampicillin and chloramphenicol in this study, because the intrinsic AmpC β-lactamases and the MexAB-OprM efflux pump were expressed in virtually all isolates (Ziha-Zarifi et al., 1999; Masuda et al., 2000; Juan et al., 2005). MICs at which 50% and 90% of the bacterial cells were inhibited (MIC_{50} and MIC_{90}) and antimicrobial susceptibility profiles against antimicrobial agents are summarized in Table 2. In the case of isolates from canine otitis externa

Table 1. Variability of extended spectrum β-lactamases and RT-qPCR specific primers used for detection of *P. aeruginosa* isolates

Primer	Sequence	Amplicon size (bp)	Reference
VEB-F	5'-CGACTTCCATTTCCCGATGC-3'	643	Strateva et al., 2007
VEB-B	5'-GGACTCTGCAACAAATACGC-3'		
PER-F	5'- AATTTGGGCTTAGGGCAGAA-3'	925	Strateva et al., 2007
PER-B	5'- ATGAATGTCATTATAAAAGC-3'		
PSE-F	5'- AATGGCAATCAGCGCTTC-3'	699	Strateva et al., 2007
PSE-B	5'- GCGCGACTGTGATGTATA-3'		
OXA-10-F	5'- TCTTTCGAGTACGGCATTAGC-3'	759	Strateva et al., 2007
OXA-10-B	5'- CCAATGATGCCCTCACTTTCC-3'		
OXA-2-F	5'- GCCAAAGGCACGATAGTTGT-3'	701	Strateva et al., 2007
OXA-2-B	5'- GCGTCCGAGTTGACTGCCGG-3'		
IMP-F	5'- GAAGGYGTTTATGTTCATAC	587	Strateva et al., 2007
IMP-B	5'- GTAMGTTTCAAGAGTGATGC		
VIM-F	5'- ATGGTGTTTGGTCGCATATC	510	Strateva et al., 2007
VIM-B	5'- TGGGCCATTCAGCCAGATC		
SPM-F	5'-CCTACAATCTAACGGCGACC-3'	649	Gales et al., 2003
SPM-R	5'-TCGCCGTGTCCAGGTATAAC-3'		
GES-F	5'-GTTTTGCAATGTGCTCAACG-3'	371	Weldhagen, 2004
GES-R	5'-TGCCATAGCAATAGGCGTAG-3'		
TEM-F	5'-ATAAAATTCTTGAAGAC-3'	1075	Jiang et al., 2006
TEM-R	5'-TTACCAATGCTTAATCA-3'		
SHV-F	5'-TGGTTATGCGTTATATTCGCC-3'	867	Jiang et al., 2006
SHV-R	5'- GCTTAGCGTTGCCAGTGCT -3'		
ampR-F	5'-AGGATTGGCGTCCTTTGTC-3'	1058	Tam et al., 2007
ampR-R	5'-CTTGAATCGCCTGCATAACC-3'		
ampE-F	5'-GCCTGGACCCGAACGAAC-3'	1231	Juan et al., 2005
ampE-R	5'-TCAGAGGAACAGCGCGCAG-3'		
ampD-F	5'-GTACGCCTGCTGGACGATG-3'	916	Juan et al., 2005
ampD-R	5'-GAGGGCAGATCCTCGACCAG-3'		
ampC-F*	5'-CAGAAGGACCAGGCACAG-3'	113	this study
ampC-R*	5'-GCGAGATAGCCGAACAGG-3'		
oprM-F*	5'-CAACGCTCGCTGTTCACC-3'	115	this study
oprM-R*	5'-TCACGGTCTGCTGGTTCC-3'		
rpoD-F*	5'-GGGCGAAGAAGGAAATGGTC-3'	178	Savli et al., 2003
rpoD-R*	5'-CAGGTGGCGTAGGTGGAGAA-3'		

*RT-qPCR

Table 2. Antimicrobial susceptibility of *P. aeruginosa* from canine and human sources

	Canine (n = 17)				Human (n = 49)			
	MIC_{50}	MIC_{90}	MIC range	% R	MIC_{50}	MIC_{90}	MIC range	% R
Ampicillin	> 1024	> 1024	> 1024	100	> 1024	> 1024	1024 –> 1024	100
Aztreonam	8	16	4–16	0	16	64	0.5–128	42.9
Cefepime	8	16	8–16	0	32	64	2–128	61.2
Ceftazidime	4	32	4–32	23.5	16	128	4–256	38.8
Chloramphenicol	256	512	128–1024	100	512	1024	128–> 1024	100
Ciprofloxacin	0.5	1	0.25–2	0	16	64	< 0.125–128	61.2
Colistin	4	16	2–32	11.8	8	32	1–64	85.7
Enrofloxacin	2	4	1–8	35.2	64	256	0.5–512	75.5
Gentamicin	4	8	2–> 1024	29.4	512	> 1 024	1–> 1024	69.4
Meropenem	1	4	0.25–8	0	8	32	0.5–64	42.9

infected with *P. aeruginosa,* 35.2% of the isolates (6/17) were resistant to enrofloxacin, 29.4% (5/17) to gentamicin, 23.5% (4/17) to ceftazidime and 11.8% (2/17) to colistin. However, all isolates from canines were sensitive to aztreonam, cefepime, ciprofloxacin and meropenem. For human isolates, the percentage of resistance to various antimicrobial agents was 85.7% (42/49) to colistin, 75.5% (37/49) to enrofloxacin, 69.4% (34/49) to gentamicin, 61.2% (30/49) to cefepime, 61.2% (30/49) to ciprofloxacin, 42.9% (21/49) to aztreonam, 42.9% (21/49) to meropenem and 38.8% (19/49) to ceftazidime. A human patient isolate was simultaneously resistant to ciprofloxacin, aztreonam, cefepime and meropenem. There was a higher percentage of resistance to aztreonam, cefepime, ceftazidime, ciprofloxacin, colistin, enrofloxacin, gentamicin, and meropenem in human clinical isolates of *P. aeruginosa,* as compared to canine isolates. This trend might be attributable to the fact that most human clinical isolates of *P. aeruginosa* were collected from nosocomial infection cases, and the therapy of all patients likely consisted of treatment with multi-antimicrobial agents.

In this study, 34.8% of the isolates (23/66) showed resistance to ceftazidime, 19 isolates from bacteremia patients and four isolates from canine otitis specimens, with MICs ranging from 32 to 256 mg/l (Table 3). However, the pattern of ceftazidime resistance in *P. aeruginosa* isolates from humans has varied considerably in previous studies. Spencer (1996) found that 95% of isolates were sensitive to ceftazidime, Panayotis et al. (1998) reported 15% isolate resistance, Shawar et al. (1999) 11.1%, Mueller-Premru and Gubina (2000) 9.1%, and De Champs et al. (2002) 6%. In contrast, Seol et al. (2002) found that 19.7% of *P. aeruginosa* isolated from canines was resistant to ceftazidime. The present study revealed that resistance to ceftazidime was 38.8% (17/49) for human isolates and 23.5% (4/17) for canine isolates. To understand the discrepancies among previous reports, all 23 ceftazidime resistance *P. aeruginosa* isolates were studied to characterize the molecular mechanisms of resistance against ceftazidime.

The ceftazidime resistance of *P. aeruginosa* isolates was also related to Ambler class A β-lactamases such as bla_{TEM}-, bla_{SHV}-, bla_{VEB}-, bla_{PER}-, or bla_{GES}-type; class B β-lactamases such as bla_{IMP}-, bla_{VIM}-, or bla_{SPM}-type; and class D β-lactamases such as bla_{OXA}-type, all of which were encountered in this study. The 23 ceftazidime resistance isolates were subjected to PCR analyses using specific primers for class A, B and D β-lactamase genes. The results revealed the following frequencies: bla_{TEM-1} 100%, bla_{PSE-1} 100%, bla_{OXA-2} 96.2%, bla_{SHV-18} 91.3%, bla_{OXA-17} 78.3%, bla_{VIM-3} 26.1%, bla_{OXA-10} 21.7% and bla_{SHV-1} 8.7%. Conversely, bla_{VEB}-, bla_{GES}-, bla_{PER}-, bla_{IMP}-, and bla_{SPM}-type ESBLs genes were not detected among these isolates (Table 3).

In this study, bla_{SHV18} was the most common SHV-type ESBL coexisting with bla_{TEM-1}, bla_{PSE-1}, $bla_{OXA\ group\ II\ (OXA-2),}$ bla_{OXA10} or bla_{OXA-17} in the ceftazidime resistance isolates of *P. aeruginosa*. In the human isolates, the most prevalent ESBLs in *P. aeruginosa* are bla_{SHV-5} and bla_{SHV-12} in Taiwan

Table 3. Characteristics of the 23 ceftazidime resistant *P. aeruginosa* isolates

Isolate[a]	MIC(μg/ml)									mRNA level[b]		*ampR*						ESBLs			
	CIP	ENR	AZT	CPM	MEM	GEN	CHL	CAZ	CAZ + EPI	*ampC*	*oprM*	12 Ala	114 Glu	135 Asp	194 Ala	237 Gly	283 Gly	288 Val	OXA	VIM	SHV	PSE
Group 1																						
H56B	16	64	128	32	16	4	128	128	128	37.80	0.45	Arg		Asn		Ala			OXA-2, OXA-17		SHV-18	PSE-1
H64B	0.25	1	128	64	16	4	128	64	32	80.20	0.67	Arg		Asn		Ala			OXA-2, OXA-17		SHV-18	PSE-1
H131B	16	1	128	64	8	4	128	256	128	68.60	0.63	Arg		Asn		Ala			OXA-2, OXA-17		SHV-18	PSE-1
H62B	32	256	64	32	32	4	256	32	16	12.70	0.72	Arg			Ser	Ala	Glu	Arg	OXA-2, OXA-17	VIM-3	SHV-18	PSE-1
NTD121	0.5	1	16	16	2	> 1024	256	32	8	10.45	2.33	Arg			Ser	Ala			OXA-10		SHV-1b	PSE-1
Group 2																						
NTD52	0.5	2	16	16	1	4	256	32	4	2.00	3.12	Arg				Ala			OXA-2, OXA-10		SHV-1b	PSE-1
H61B	32	256	64	32	32	512	256	32	2	4.18	3.5	Arg				Ala	Glu		OXA-2, OXA-17		SHV-18	PSE-1
H270B	64	512	128	128	32	> 1024	512	256	32	1.40	3.95	Arg				Ala			OXA-2, OXA-10	VIM-3	SHV-18	PSE-1
H307B	16	64	64	64	32	512	1024	64	8	25.55	3.68	Arg				Ala			OXA-2, OXA-17		SHV-18	PSE-1
H253B	16	64	32	32	32	512	512	64	8	0.10	3.42	Arg				Ala			OXA-2, OXA-17	VIM-3	SHV-18	PSE-1
Group 3																						
H468B	32	128	16	64	32	256	256	32	32	0.34	0.32	Arg				Ala	Glu	Arg	OXA-2, OXA-10	VIM-3	SHV-18	PSE-1
H471B	32	128	16	32	16	> 1024	1024	32	16	0.06	0.33	Arg				Ala	Glu		OXA-2, OXA-17	VIM-3	SHV-18	PSE-1
H236B	0.5	4	8	128	64	> 1024	256	32	32	0.04	0.32	Arg				Ala			OXA-2, OXA-17	VIM-3	SHV-18	PSE-1
H450B	32	128	32	32	16	512	256	32	16	0.6	0.32	Arg				Ala	Glu	Arg	OXA-2, OXA-10		SHV-18	PSE-1
H410B	8	32	16	64	2	> 1024	128	256	256	0.1	0.43	Arg				Ala			OXA-2, OXA-17		SHV-18	PSE-1
H517B	0.25	1	64	128	1	1	1024	128	64	0.31	0.41	Arg				Ala			OXA-2, OXA-17		SHV-18	PSE-1
NTD91	0.25	2	8	8	0.5	4	256	32	16	0.17	0.59	Arg				Ala			OXA-2, OXA-17		SHV-18	PSE-1
NTD106	0.25	2	8	8	1	2	128	32	16	1.23	0.78	Arg				Ala			OXA-2, OXA-17		SHV-18	PSE-1
H364B	16	64	8	64	2	> 1024	256	64	64	0.04	0.61	Arg				Ala			OXA-2, OXA-17		SHV-18	PSE-1
H430B	16	64	8	32	4	> 1024	256	32	32	0.01	0.37	Arg	Ala			Ala	Glu	Arg	OXA-2, OXA-17		SHV-18	PSE-1
H144B	8	32	8	32	8	256	256	256	128	0.04	0.89	Arg				Ala			OXA-2, OXA-17		SHV-18	PSE-1
H182B	8	128	8	32	8	512	256	128	128	0.02	0.42	Arg				Ala			OXA-2, OXA-17		SHV-18	PSE-1
H248B	16	64	32	32	4	512	1024	128	128	0.04	0.5	Arg				Ala			OXA-2, OXA-17		SHV-18	PSE-1

CIP = ciprofloxacin; ENR = enrofloxacin; AZT = aztreonam; CPM = cefepime; MEM = meropenem; GEN = gentamicin; CHL = chloramphenicol; CAZ = ceftazidime; EPI = PAβN; [a]H = human; NTD = canine sample; [b]the ratio of gene expression derived from the target gene (*ampC* and *oprM*) and *P.aeruginosa* ATCC 27853

(Yu et al., 2006). However, in the current study, we found that the bla_{SHV-18}-producing member of *P. aeruginosa* occurred in both canine and human isolates. Twenty-one isolates were positive for the bla_{SHV-18} gene (Table 3), localized in plasmids ranging from 35 to 50 kb, as detected by Southern blot hybridization. Two isolates (NTD 91 and NTD106) of ceftazidime resistance *P. aeruginosa* from canines carried bla_{SHV-18}. The clinical history of the canine isolates did not include the use of any expanded-spectrum cephalosporins, though the canines might have been previously treated by other clinical surgeons.

The OXA-10 derivatives confer greater resistance to ceftazidime than to cefepime. Among the extended spectrum derivatives of OXA-10, the OXA-11, -14, and -16 derivatives confer a high level of resistance to ceftazidime (MIC > 128 mg/l). By contrast, OXA-10 and OXA-17 has minimal effects on the MIC of ceftazidime. In this study, all 23 ceftazidime resistance isolates produced OXA-10 or -17, and showed marginal resistance (Strateva et al., 2007). In this study, the human isolates of H62B, H236B, H253B, H270B, H468B, and H471B produced a bla_{VIM-3} metallo-β-lactamase and conferred increased resistance to ceftazidime (MICs, 32–256 mg/l). The bla_{VIM}-positive isolates showed consistent high-level resistance to almost all broad-spectrum cephalosporines, including ceftazidime and cefoperazone-sulbactam (MICs, ≥ 128 mg/l) (Senda et al., 1996; Yan et al., 2001).

The presence of mutations responsible for the AmpC resistance phenotypes was subsequently investigated by PCR, and the genes for *ampD*, *ampE*, *ampR* as well as the *ampC-ampR* intergenic region were sequenced for the 23 ceftazidime resistance isolates. None of the resistance isolates contained mutations in the *ampC-ampR* intergenic region compared with their isogenic susceptible isolates. However, 23 ceftazidime resistance isolates had DNA mutations in the *ampD* structural gene, resulting in the substitution of nine amino acids at positions 10 (Val to Gly), 11 (Arg to Leu), 44 (Gln to His), 118 (Glu to Gly), 136 (Ala to Val), 148 (Gly to Ala), 174 (Trp to Cys), 175 (Ser to Leu) and 183 (Asp to Tyr) (Table 4). In the *ampR* structural gene, seven amino acids differed at positions 12 (Ala to Arg), 114 (Glu to Ala), 135 (Asp to Asn), 194 (Ala to Ser), 237 (Gly to Ala), 283 (Gly to Glu), and 288 (Val to Arg) (Table 3). In the *ampE* structural gene, eight amino acid changes were detected at positions 20 (Arg to Gln), 23 (Gln to Pro), 33 (Ser to Gly), 34 (Leu to Gln), 69 (Ser to Pro), 122 (Ile to Val), 129 (Glu to Asp), and 136 (Gly to Ser) (Table 5).

Many studies have shown that ceftazidime resistance isolates from humans are characterised by the overproduction of AmpC or OprM (Campbell et al., 1997; Li et al., 2000; Masuda et al., 2000; Bagge et al., 2002; Livermore et al., 2002; Henrichfreise et al., 2007; Tam et al., 2007). RT-*q*PCR was performed for quantitative analysis of mRNA expression of the *ampC* and *oprM* genes. Total cellular mRNA was prepared from the 23 *P. aeruginosa* isolates. The results for the relative expression of the *ampC* and *oprM* genes are summarized in Table 3. Group 1 isolates were characterized by increased *ampC* expression. Three ceftazidime resistance isolates (H56B, H64B, H131B from humans) were char-

Table 4. Point mutations detected in *ampD* from canine and human isolates of *P. aeruginosa*

10 Val	11 Arg	44 Gln	118 Glu	136 Ala	148 Gly	174 Trp	175 Ser	183 Asp	Canine isolates	Human isolates	*ampC* expression	Ceftazidime MIC range
									3	7	0.01–3	4–32
		His			Ala			Tyr	0	7	0.02–0.1	4–256
	Leu				Ala			Tyr	4	2	0.21–3.57	4–8
					Ala			Tyr	4	8	1.23–80.2	4–256
					Ala				3	17	0.04–12.7	4–128
					Ala		Leu		3	1	0.31–10.45	4–32
			Gly						0	2	0.23–4.17	32
				Val	Ala				0	3	0.04–0.05	4–32
	Leu				Ala	Cys		Tyr	0	1	0.27	16
Gly					Ala				0	1	0.06	32

Table 5. Point mutations detected in *ampE* from canine and human isolates of *P. aeruginosa*

20 Arg	23 Gln	33 Ser	34 Leu	69 Ser	122 Ile	129 Glu	136 Gly	Canine isolates	Human isolates	*ampC* expression[a] (fold-difference)	Ceftazidime MIC range
		Gly		Pro	Val		Ser	1	13	0.04–12.7	4–32
		Gly		Pro		Asp		1	1	0.13–0.37	4–8
				Pro		Asp		0	4	0.37–0.89	4–32
		Gly		Pro				8	21	0.31–2.08	4–256
		Gly		Pro			Ser	1	2	0.27–25.55	8–64
				Pro			Ser	0	4	0.18–0.32	4–32
			Gln	Pro				2	1	0.36–1.4	4–8
Gln		Gly		Pro				1	0	2	32
	Pro	Gly		Pro	Val		Ser	0	1	0.29	4
				Pro				3	2	0.59–1.54	4–32

[a]transcriptional expression of *ampC* as measured by RT-qPCR. Values represent the difference (*n*-fold) in gene expression relative to wild-type strain *P. aeruginosa* ATCC 27853

acterized by a point mutation in AmpR (Asp135 – Asn), which caused a 37.80- to 80.20-fold increase in *ampC* mRNA levels, as compared to the levels in *P. aeruginosa* ATCC 27853 (Table 3). The other two isolates (NTD 121 from canine; H62B from human) from Group 1 also demonstrated a point mutation in AmpR (Als194 – Ser), which resulted in a 10.45- to 12.70-fold overproduction of AmpC (Table 3). A novel amino acid substitution in position 194 is thought to have caused the overexpression of *ampC*.

Bratu et al. (2007) have demonstrated that increased *ampC* expression is associated with major deletions that affect *ampD*, and Langaee et al. (2000) have shown that mutations within the structural gene of *ampD* can lead to *ampC* overexpression. These studies also found that increased β-lactam MICs in organisms with inducible *ampC* and *ampE* genes can modulate *ampC* repression in hyperproducing strains in the absence of inducers. Nevertheless, Bratu et al. (2007) emphasize that most of their 33 clinical isolates had one or two amino acid substitutions involving *ampD* and *ampE*, which do not seem to affect expression of *ampC*. A variety of limited levels of *ampC* expression were found from the isolates of *ampD* mutations (Table 4) and *ampE* mutations (Table 5), although a few isolates had 80.2 (H64B), 12.7 (H62D) and 10.45 (NTD121) fold-difference for *ampD* mutations (Table 4); and 25.55 (H307B) and 12.7 (H62B) for *ampE* mutations (Table 5). When comparing the same isolates for *ampR* effect on the levels of *ampC* expression (Table 3) with the effect of *ampD* (Table 4) and *ampE* (Table 5), we attribute most of this AmpC overproduction to the point mutation in AmpR even if there was observed a combined effect of *ampR, ampD and ampE* in the same isolates. In the present study, the multiple mutations observed in AmpD and AmpE require further study in order to explain the differences in transcription levels, consistent with the report of Bratu et al. (2007). Moreover, these results show that ceftazidime resistance mediated by AmpC overproduction could be attributed to mutations in the *ampR* gene.

Group 2 isolates were characterized by increased expression of OprM. The transcriptional level of *oprM* is shown in Table 3. Aires et al. (1999) reported up-regulation of the expression of the efflux systems MexAB and MexXY in the *P. aeruginosa* strains. Five of the 23 (21.7%) ceftazidime resistance isolates, one isolate (NTD 52) from canine and four isolates (H61B, H270B, H307B, and H253B) from humans, showed over-expression of *oprM* mRNA, which compared with that in *P. aeruginosa* ATCC 27853, ranged between a 3.12- to 3.95-fold increase. The overproduction of OprM was absent in ciprofloxacin (MIC 0.5 mg/l) and meropenem (MIC 1 mg/l) resistance in canine isolate (NTD 52), but it was detected in very common resistance isolates (ciprofloxacin MIC 16–64 and meropenem MIC 32 mg/l), enrofloxacin, aztreonam, gentamicin, and chloramphenicol in human isolates (Table 3).

The results showed that overproduction of OprM increased the MICs of fluoroquinolones, aminoglycosides and aztreonam, consistent with findings described elsewhere (Li et al., 2000; Masuda et al., 2000).

The efflux pump inhibition assay in the absence or presence of the efflux inhibitor PAβN, as shown in Table 3, showed a significant decrease (8- to 16-fold) in ceftazidime MIC upon addition of PAβN to Group 2. For Group 1 and 3, resistance to ceftazidime was found uniquely in the presence of PAβN. In the present study, 21.7% (5/23) of the ceftazidime-resistant *P. aeruginosa* isolates were related to overproduction of the drug efflux pump. These results indicate that the clinical use of β-lactams and/or other antimicrobial agents as therapy for both canines and humans might impose a strong selective pressure for the emergence of resistant bacteria.

Acknowledgments

We thank the Song-shan Armed Forces General Hospital Department of Clinical Pathology and the National Taiwan University Veterinary Hospital for generously providing clinical *P. aeruginosa* isolates.

REFERENCES

Aires JR, Kohler T, Nikaido H, Plesiat P (1999): Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. Antimicrobial Agents and Chemotherapy, 43, 2624–2628.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997): Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research, 25**,** 3389–3402.

Bagge N, Ciofu O, Hentzer M, Campbell JIA, Givskov M, Hoiby N (2002): Constitutive high expression of chromosomal β-lactamase in *Pseudomonas aeruginosa* caused by a new insertion sequence (IS*1669*) located in *ampD*. Antimicrobial Agents and Chemotherapy, 46**,** 3406–3411.

Berthelot P, Grattard F, Mallaval FO, Ros A, Lucht F, Pozzetto B (2005): Epidemiology of nosocomial infections due to *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Stenotrophomonas maltophilia*. Pathologie Biologie, 53, 341–348.

Bratu S, Landman D, Gupta J, Quale J (2007)**:** Role of AmpD, OprF and penicillin-binding proteins in beta-lactam resistance in clinical isolates of *Pseudomonas aeruginosa*. Journal of Medical Microbiology, 56, 809–814.

Campbell JIA, Ciofu O, Hoiby N (1997): *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis have different β-lactamase expression phenotypes but are homogeneous in the *ampC-ampR* genetic region. Antimicrobial Agents and Chemotherapy, 41**,** 1380–1384.

CLSI (Clinical and Laboratory Standards Institute) (2006): Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Document M7-A7. Wayne, PA.

Cole LK, Kwochka KW, Kowalski JJ, Hillier A (1998): Microbial flora and antimicrobial susceptibility patterns from canines with otitis media. Journal of the American Veterinary Medical Association, 212, 534–538.

De Champs C, Poirel L, Bonnet R, Sirot D, Chanal C, Sirot J, Nordmann P (2002): Prospective survey of beta-lactamases produced by ceftazidime-resistant *Pseudomonas aeruginosa* isolated in a French hospital in 2000. Antimicrobial Agents and Chemotherapy, 46, 3031–3034.

El Amary EB, Chamot E, Auckenthaler R, Pechere JC, Van Delden C (2001): Influence of previous exposure to antibiotic therapy on the susceptibility pattern of *Pseudomonas aeruginosa* bacteremic isolates. Clinical Infectious Diseases, 33, 1859–1864.

Gales AC, Menezes LC, Silbert S, Sader HS (2003): Dissemination in distinct Brazilian regions of an epidemic carbapenem-resistant *Pseudomonas aeruginosa* producing SPM metallo-beta-lactamase. The Journal of Antimicrobial Chemotherapy, 52, 699–702.

Griffin CE (1993): Otitis externa and otitis media. In: Griffin CE, Kwochka KW, MacDonald JM (eds.): Current Veterinary Dermatology. Mosby Year Book, St. Louis. 245–262.

Henrichfreise B, Wiegand I, Pfister W, Wiedemann B (2007): Resistance mechanisms of multiresistant *Pseudomonas aeruginosa* strains from Germany and correlation with hypermutation. Antimicrobial Agents and Chemotherapy, 51, 4062–4070.

Jiang X, Zhang Z, Li M, Zhou D, Ruan F, Lu Y (2006): Detection of extended-spectrum beta-lactamases in clinical isolates of *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy, 50, 2990–2995.

Juan C, Macia MD, Gutierrez O, Vidal C, Perez JL, Oliver A (2005): Molecular mechanisms of β-lactam resistance mediated by AmpC hyperproduction in *Pseudomonas aeruginosa* clinical strains. Antimicrobial Agents and Chemotherapy, 49**,** 4733–4738.

Kuga A, Okamoto R, Inoue M (2000): *ampR* gene mutations that greatly increase class C β-lactamase activity

in *Enterobacter cloacae*. Antimicrobial Agents and Chemotherapy, 44, 561–567.

Langaee TY, Gagnon L, Huletsky A (2000): Inactivation of the *ampD* gene in *Pseudomonas aeruginosa* leads to moderate-basal-level and hyperinducible AmpC β-lactamase expression. Antimicrobial Agents and Chemotherapy, 44, 583–589.

Lindberg F, Lindquist S, Normark S (1987): Inactivation of the *ampD* gene causes semiconstitutive overproduction of the inducible *Citrobacter freundii* β-lactamase. Journal of Bacteriology, 169**,** 1923–1928.

Li XZ, Ma D, Livermore DM, Nikaido H (1994): Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: active efflux as a contributing factor to beta-lactam resistance. Antimicrobial Agents and Chemotherapy, 38**,** 1742–1752.

Li XZ, Zhang L, Poole K (2000): Interplay between the MexA-MexB-OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resistance of *Pseudomonas aeruginosa*. The Journal of Antimicrobial Chemotherapy, 45, 433–436.

Livak KJ, Schmittgen TD (2001): Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. Methods, 25, 402–408.

Livermore DM (2002): Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? Clinical Infectious Diseases, 34, 634–640.

Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T (2000): Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-OprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy, 44**,** 3322–3327.

Mesaros N, Glupczynski Y, Avrain L, Caceres NE, Tulkens PM, Van Bambeke F (2007): A combined phenotypic and genotypic method for the detection of Mex efflux pumps in *Pseudomonas aeruginosa*. The Journal of Antimicrobial Chemotherapy, 59, 378–386.

Mueller-Premru M, Gubina M (2000): Serotype, antimicrobial susceptibility and clone distribution of *Pseudomonas aeruginosa* in a University hospital. Zentralblatt fur Bakteriologie, 8, 857–867.

Nordmann P, Guibert M (1998): Extended-spectrum β-lactamase in *Pseudomonas aeruginosa.* The Journal of Antimicrobial Chemotherapy, 42, 128–131.

Panayotis TT, Gennimata V, Maniatis AN, Fock C, Legakis NJ, The Greek Pseudomonas aeruginosa Study Group (1998): Emergence of multidrug resistance in ubiquitous and dominant *Pseudomonas aeruginosa* serogroup O:11. Journal of Clinical Microbiology, 36, 897–901.

Peterson AD, Walker RD, Bowman MM, Schott HC, Rosser EJ (2002): Frequency of isolation of and antimicrobial susceptibility patterns of *Staphylococcus intermedius* and *Pseudomonas aeruginosa* isolates from canine skin and ear samples over a 6-year period (1992–1997). Journal of the American Animal Hospital Association, 38, 407–413.

Sambrook J, Fritsch EF, Maniatis T (1989): Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, NY. 9, 14.

Savli H, Karadenizli A, Kolayli F, Gundes S, Ozbek U, Vahaboglu H (2003): Expression stability of six housekeeping genes*:* a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. Journal of Medical Microbiology, 52, 403–408.

Scott DW, Miller WH, Griffin CE (2001): Muller and Kirk's Small Animal Dermatology. 6th ed. WB Saunders, Philadelphia. 729–740.

Senda K, Arakawa Y, Nakashima K, Ito H, Ichiyama S, Shimokata K, Kato N, Ohta M (1996): Multifocal outbreaks of metallo-β-lactamase-producing *Pseudomonas aeruginosa* resistant to broad-spectrum-β-lactams, including carbapenems. Antimicrobial Agents and Chemotherapy, 40, 349–353.

Seol B, Naglic T, Madic J, Bedekovic M (2002): *In vitro* antimicrobial susceptibility of 183 *Pseudomonas aeruginosa* strains isolated from canines to selected antipseudomonal agents. Journal of Veterinary Medicine B, Infectious Diseases and Veterinary Public Health, 49, 188–192.

Shawar R, MacLeod MDL, Garber RL, Burns JL, Stapp JR, Clausen CR, Tanaka SK (1999): Activities of tobramycin and six other antibiotics against *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Antimicrobial Agents and Chemotherapy, 43**,** 2877–2880.

Spencer RC (1996): An 8 year microbe base survey of the epidemiology, frequency and antibiotic susceptibility of *Pseudomonas aeruginosa* hospital isolates in United Kingdom. The Journal of Antimicrobial Chemotherapy, 37, 295–301.

Stapleton P, Shannon K, Phillips I (1995): DNA sequence differences of *ampD* mutants of *Citrobacter freundii*. Antimicrobial Agents and Chemotherapy, 39, 2494–2498.

Strateva T, Ouzounova-Raykova V, Markova B, Todorova A, Marteva-Proevska Y, Mitov I (2007): Problematic clinical isolates of *Pseudomonas aeruginosa* from the university hospitals in Sofia, Bulgaria: current status of antimicrobial resistance and prevailing resistance mechanisms. Journal of Medical Microbiology, 56, 956–963.

Tam VH, Schilling AN, LaRocco MT, Gentry LO, Lolans K, Quinn JP, Garey KW (2007): Prevalence of AmpC

over-expression in bloodstream isolates of *Pseudomonas aeruginosa*. Clinical Microbiology and Infection, 13, 413–418.

Weldhagen GF (2004): Sequence-selective recognition of extended-spectrum beta-lactamase GES-2 by a competitive, peptide nucleic acid-based multiplex PCR assay. Antimicrobial Agents and Chemotherapy, 48, 3402–3406.

Yan JJ, Hsueh PR, Ko WC, Luh KT, Tsai SH, Wu HM, Wu JJ (2001): Metallo-beta-lactamases in clinical *Pseudomonas* isolates in Taiwan and identification of VIM-3, a novel variant of the VIM-2 enzyme. Antimicrobial Agents and Chemotherapy, 45, 2224–2228.

Yu WL, Chuang YC, Walther-Rasmussen J (2006): Extended-spectrum beta-lactamases in Taiwan: epidemiology, detection, treatment and infection control. Journal of Microbiology, Immunology, and Infection, 39, 264–277.

Ziha-Zarifi I, Llanes C, Kohler T, Pechere JC, Pleaist P (1999): *In vivo* emergence of multidrug resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. Antimicrobial Agents and Chemotherapy, 43, 287–291.

Received: 2010–02–10
Accepted after corrections: 2010–04–30

Corresponding Author:

Shao-Kuang Chang, National Taiwan University, Graduate Institute of Veterinary Medicine, No. 1, Sec. 4, Roosevelt Rd., Taipei 106, Taiwan
Tel. +886 2 33663864, Fax +886 2 23661475, E-mail: changsk@ntu.edu.tw