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Investigation of β-Lactamase-producing Multidrug-resistant *Pseudomonas aeruginosa* Isolated from Non-Tertiary Care Hospitals in Korea

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Abstract A total of 2,280 nonduplicate clinical isolates of *Pseudomonas aeruginosa*, obtained nationwide from Korean non-tertiary care hospitals from 2002 to 2005, were identified and their susceptibilities to aminoglycosides, antipseudomonal penicillins, carbapenems, cephalosporins, monobactams, and quinolones were studied, together with their production of β-lactamases. Using disk diffusion and minimum inhibitory concentration tests, it was found that 2.9% of isolates were multidrug-resistant (MDR) *P. aeruginosa*. An EDTA-disk synergy test, PCR amplification with specifically designed primers, and direct sequencing of the PCR products showed that the bla_{OXA-10}, bla_{VIM-2}, bla_{OXA-2}, bla_{OXA-17}, bla_{PER-1}, bla_{SHV-12}, and bla_{IMP-1} genes were carried by 34.3%, 26.9%, 3.0%, 3.0%, 1.5%, 1.5%, and 1.5% of 67 MDR *P. aeruginosa* isolates, respectively. The prevalence of MDR *P. aeruginosa* was three-fold higher, compared with that from the United States. More than two types of β-lactamase genes were carried by 10.4% of isolates. The most prevalent β-lactamase genes were bla_{VIM-2} and bla_{OXA-10}. This study is the first description of MDR *P. aeruginosa* from non-tertiary care hospitals in Korea and the coexistence of the bla_{OXA-10} gene with bla_{VIM-2}, bla_{IMP-1}, or bla_{PER-1} in these clinical isolates.

Keywords: Multidrug resistance, *Pseudomonas aeruginosa*, VIM-2, OXA-10, PER-1

Pseudomonas aeruginosa is an opportunistic human pathogen that constitutes one of the most prevalent causes of nosocomial infections in the world [2]. Unfortunately, resistance to available antipseudomonal agents (amikacin, ceftazidime, ciprofloxacin, gentamicin, imipenem, and piperacillin) continues to increase, jeopardizing the selection of appropriate treatments, with a consequent increase in morbidity and mortality among patients infected with this pathogen [15]. The prevalence of multidrug-resistant (MDR) *P. aeruginosa* has increased over the past decade and has become a major concern in the care of hospitalized patients [6, 7, 16–18]. Until now, most investigations of antimicrobial resistance and MDR in *P. aeruginosa* have been performed in the intensive care units (ICUs) of tertiary care hospitals. Only a few cases have been reported in community hospitals [1, 8, 22, 24]. The rate of MDR *P. aeruginosa* isolated in ICU patients in the United States increased from 4% in 1993 to 14% in 2002 [18]. According to the Global SENTRY Antimicrobial Surveillance Program (1997–1999) report, MDR *P. aeruginosa*, which is defined as being resistant to piperacillin, imipenem, ceftazidime, and gentamicin, constituted 3% of isolates [7]. The Surveillance Network Database-U.S.A. (Focus Technologies, Herndon, VA, U.S.A.) documented that 16% of isolates were resistant to more than three antipseudomonal agents, and more than 1% of isolates were resistant to all agents [16]. The incidence of resistance to β-lactam antimicrobial agents among Gram-negative pathogens in Korea is an increasing problem, and MDR *P. aeruginosa* has many resistance mechanisms directed against β-lactam antimicrobial agents. β-Lactamase is the most prevalent mechanism, and several classes have recently been identified in *P. aeruginosa*: the classes A, B, and D extended-spectrum β-lactamases (ESBLs) [26]. However, they have so far been studied only in tertiary care hospital isolates, and there have been few studies of MDR strains [12, 14]. This study was performed to investigate the prevalence of MDR *P. aeruginosa* in community hospitals nationwide, and to estimate the prevalence of the Ambler classes A, B, and D β-lactamases produced by MDR *P. aeruginosa*.

A total of 2,280 nonduplicate clinical isolates of *P. aeruginosa* were obtained from 2,636 patients hospitalized at 373 non-tertiary care hospitals in all provinces in Korea, from January 2002 to December 2005. The isolates were

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identified with conventional techniques [10] and/or using the Vitek GNI card (bioMérieux Vitek Inc., Hazelwood, MO, U.S.A.). *P. aeruginosa* ATCC 27853 was used as the quality-control strain for antimicrobial susceptibility testing. Antimicrobial susceptibility was evaluated with the disk diffusion test, which was conducted in accordance with the instruction of the Clinical and Laboratory Standards Institute (CLSI) [4], using disks from BBL (Cockeysville, MD, U.S.A.) impregnated with aztreonam (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), cefepime (30 μg), gentamicin (10 μg), imipenem (10 μg), piperacillin (100 μg), or ticarcillin/clavulanic acid (75 μg and 10 μg, respectively). Minimum inhibitory concentrations (MICs) were determined with the agar dilution method on Muller-Hinton agar plates (Becton-Dickinson, Sparks, MD, U.S.A.) [4]. *P. aeruginosa* was defined as MDR if the organism was resistant to gentamicin, ciprofloxacin, ticarcillin/clavulanic acid, ceftazidime, and imipenem. To identify the MDR *P. aeruginosa* strain with the most resistant profile, these criteria for multidrug resistance in *P. aeruginosa* were at the more extreme end (resistance to five antimicrobial agents) of the spectrum of antimicrobial resistance profiles used for the definition of MDR, relative to those of Falagas *et al.* [5] and Sekiguchi *et al.* [22]. The prevalence of *P. aeruginosa* resistant to ceftazidime, cefepime, aztreonam, and imipenem was 12.8%, 11.8%, 14.5%, and 15.0%, respectively. Of 1,038 clinical isolates resistant to ceftazidime, cefepime, aztreonam, and/or imipenem, 67 MDR *P. aeruginosa* were resistant to five antimicrobial categories: aminoglycosides (gentamicin), antipseudomonal penicillins (ticarcillin/clavulanic acid), carbapenems (imipenem), cephalosporins (ceftazidime), and quinolones (ciprofloxacin). The nationwide prevalence of MDR *P. aeruginosa* in Korean non-tertiary care hospitals was 2.9% (67 of 2,280). This is similar to the prevalence (3.6%) of such strains from Latin America [7], but higher than those (1.0% and 0.9%, respectively) from the United States [16] and Canada [7]. The rate of MDR *P. aeruginosa* isolated in ICU patients in the U.S.A. increased from 4% in 1993 to 14% in 2002 [18]. Although the prevalence of MDR *P. aeruginosa* in non-tertiary care hospitals was lower than that in ICU patients, the prevalence will be increased because of the selection pressure by antimicrobial usage. Thus, the infectious disease control of MDR *P. aeruginosa* is required. Forty-nine isolates (73%) of 67 β-lactamase-producing MDR *P. aeruginosa* isolates were resistant to all nine antimicrobial agents tested.

Microbiological testing for metallo-β-lactamase (MBL) activity was conducted with an EDTA-disk synergy test [9, 11], modified as follows. An overnight culture of the test strain was grown to the turbidity of the McFarland No. 0.5 tube, and then inoculated onto a Muller-Hinton agar plate. After the culture had been dried, an imipenem disk (BBL) and a blank filter paper disk were positioned 15 mm apart, from center to center. Then, 10 μl of 0.5 M EDTA solution was applied to the blank disk, resulting in a concentration of about 1.5 mg/disk. After overnight incubation, the presence of an enlarged inhibition zone was interpreted as positive, revealing the inactivation of MBL activity by EDTA. Sixty-seven MDR *P. aeruginosa* isolates were analyzed with the EDTA-disk synergy test. Metallo-β-lactamase generation (positive EDTA-disk synergy test) was detected in 18 isolates.

To investigate the production of β-lactamases in these 67 MDR *P. aeruginosa* isolates, the β-lactamase genotypes

Table 1. Nucleotide sequences of the oligonucelotides used for PCR amplifications and sequencing of bla_{PER}-, bla_{SHV}-, bla_{VIM}-, bla_{IMP}-, and bla_{OXA}-type genes.

Gene name	Primer name[a]	Sequence	Amplicon size (bp)	Reference
bla_{PER}	P1	5'-ATGAATGTCATTATAAAAGC-3'	924	[20]
	P2	5'-AATTTGGGCTTAGGGCAGAA-3'	(P1/P2)[b]	
bla_{SHV}	S1	5'-GGGTTATTCTTATTTGTCGCT-3'	929	[14]
	S2	5'-TAGCGTTGCCAGTGCTCG-3'	(S1/S2)	
	S3	5'-AGATCCACTATCGCCAGCA-3'	231	[14]
	S4	5'-TTCAGTTCCGTTTCCCAGC-3'	(S3/S4)	
bla_{VIM}	V1	5'-ATGTTCAAACTTTTGAGTAGTAAG-3'	801	[21]
	V2	5'-CTACTCAACGACTGAGCG-3'	(V1/V2)	
	V3	5'-AGATCCACTATCGCCAGCA-3'	601	This study
	V4	5'-TTCAGTTCCGTTTCCCAGC-3'	(V3/V4)	
bla_{IMP}	I1	5'-CATGGTTTGGTGGTTCTTGT-3'	448	[13]
	I2	5'-ATAATTTGGCGGACTTTGGC-3'	(I1/I2)	
bla_{OXA}	O1	5'-GTCTTTCGAGTACGGCATTA-3'	720	[25]
	O2	5'-ATTTTCTTAGCGGCAACTTAC-3'	(O1/O2)	
	O3	5'-GTGGCAGACGAACGCCAA-3'	580	This study
	O4	5'-CCACTCAACCCATCCTACCC-3'	(O3/O4)	

Primers are consensus sequences of the *bla* (β-lactamase) genes of nucleotide sequence accession numbers and reference reports.
[a]Primers P1, S1, S3, V1, V3, I1, O1, and O3 are identical to the leading strand; primers P2, S2, S4, V2, V4, I2, O2, and O4 are identical to the lagging strand.
[b]Primer pair for PCR amplification.

of the 67 isolates were analyzed using specifically designed primers (Table 1). PCR amplifications and the sequencing of PCR products were carried out as described previously [3, 13, 14, 20, 21, 23] and the annealing temperatures for the PCRs with primer pairs V3/V4, O1/O2, and O3/O4 were 50°C, 50°C, and 52°C, respectively. Using the designed primers, β-lactamase genes in 40 among 67 MDR *P. aeruginosa* isolates were detected. The MICs of aminoglycosides, antipseudomonal penicillins, carbapenems, cephalosporins, monobactams, and quinolones for 40 β-lactamase-producing MDR *P. aeruginosa* isolates are listed in Table 2. All isolates were resistant to gentamicin, ciprofloxacin, ticarcillin/

Table 2. Profiles of multidrug-resistant and β-lactamase-producing *P. aeruginosa* strains isolated in Korean nationwide community hospitals from 2002 to 2005.

Strain	Type of specimen	MIC (mg/ml) of different β-lactams[a]									Genotype of β-lactamase
		PIP	TIM	CAZ	CTX	FEP	GM	AZT	CIP	IMP	
PA5172	Pus	512	>512	256	>512	256	>512	>512	16	32	bla_{SHV-12}
PA3648	Urine	512	512	256	>512	256	>512	64	64	>512	bla_{VIM-2}
PA3801	Urine	256	>512	128	>512	128	>512	64	32	512	bla_{VIM-2}
PA3817	Urine	128	>512	32	>512	16	128	8	8	32	bla_{VIM-2}
PA3871	Urine	64	>512	32	512	16	256	8	8	16	bla_{VIM-2}
PA3987	Urine	256	>512	128	>512	32	256	128	4	64	bla_{VIM-2}
PA4069	Urine	256	>512	256	>512	64	>512	128	256	512	bla_{VIM-2}
PA4101	Urine	512	>512	128	>512	128	>512	64	256	>512	bla_{VIM-2}
PA4152	Urine	256	>512	64	>512	64	>512	64	256	>512	bla_{VIM-2}
PA4204	Urine	256	>512	256	>512	64	256	128	8	64	bla_{VIM-2}
PA5158	Sputum	256	>512	64	>512	64	>512	64	256	>512	bla_{VIM-2}
PA05-432	Urine	512	>512	64	64	64	>512	32	128	512	bla_{VIM-2}
PA05-596	Urine	256	>512	64	64	64	>512	32	64	256	bla_{VIM-2}
PA3759	Sputum	32	256	128	128	8	>512	32	8	32	bla_{OXA-2}
PA5225	Sputum	256	>512	32	>512	16	>512	32	16	16	bla_{OXA-2}
PA2929	Urine	256	256	32	512	16	>512	32	8	16	bla_{OXA-10}
PA3660	Urine	128	128	32	512	64	>512	16	16	8	bla_{OXA-10}
PA3661	Urine	256	256	128	512	16	>512	32	8	8	bla_{OXA-10}
PA3790	Sputum	256	256	32	512	16	>512	32	8	16	bla_{OXA-10}
PA3820	Sputum	512	512	64	>512	16	>512	32	16	16	bla_{OXA-10}
PA4079	Urine	8	256	64	>512	16	>512	16	16	32	bla_{OXA-10}
PA4113	Urine	256	>512	128	>512	64	>512	4	64	64	bla_{OXA-10}
PA4122	Urine	512	>512	32	>512	128	16	32	128	16	bla_{OXA-10}
PA4190	Pus	512	512	128	>512	128	>512	64	32	16	bla_{OXA-10}
PA4259	Sputum	256	512	32	>512	128	>512	16	16	16	bla_{OXA-10}
PA4389	Sputum	512	512	32	>512	64	>512	16	8	16	bla_{OXA-10}
PA5168	Sputum	256	256	64	512	16	>512	32	>128	16	bla_{OXA-10}
PA5969	Urine	256	>512	64	>512	128	16	128	16	16	bla_{OXA-10}
PA5979	Wound	256	>512	32	256	32	>512	32	8	16	bla_{OXA-10}
PA05-320	Urine	64	>512	128	>512	256	>512	16	16	128	bla_{OXA-10}
PA05-495	Urine	256	>512	128	>512	128	>512	64	32	8	bla_{OXA-10}
PA3889	Sputum	32	512	64	256	64	64	4	8	8	bla_{OXA-17}
PA4216	Pus	64	128	64	32	8	64	16	8	16	bla_{OXA-17}
PA5175	Urine	256	>512	>512	>512	>512	64	4	16	64	bla_{IMP-1}, bla_{OXA-10}
PA3422	Urine	512	>512	256	>512	64	256	32	64	>512	bla_{VIM-2}, bla_{OXA-10}
PA3701	Urine	256	>512	64	512	32	>512	16	32	512	bla_{VIM-2}, bla_{OXA-10}
PA4107	Urine	256	>512	128	>512	64	>512	64	256	>512	bla_{VIM-2}, bla_{OXA-10}
PA5874	Urine	256	>512	64	>512	64	>512	64	32	16	bla_{VIM-2}, bla_{OXA-10}
PA5926	Sputum	256	>512	32	>512	128	>512	16	16	32	bla_{VIM-2}, bla_{OXA-10}
PA4106	Urine	256	>512	64	512	32	>512	32	8	16	bla_{PER-1}, bla_{VIM-2}, bla_{OXA-10}
ATCC 27853		2	16	16	1	1	0.5	0.5	0.5	<0.5	

[a]Abbreviations: PIP, piperacillin; TIM, ticarcillin-clavulanic acid; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; GM, gentamicin; CIP, ciprofloxacin; ATM, aztreonam; IMP, imipenem.

clavulanic acid, ceftazidime, and imipenem, but displayed varying degrees of resistance to the other antimicrobial agents. Resistance to cefotaxime (MIC ≥64 μg/ml) was observed in 39 of 40 isolates (97.5%), and resistance to piperacillin (MIC ≥128 μg/ml) was observed in 35 of 40 isolates (87.5%). Resistance to cefepime (MIC ≥32 μg/ml) was observed in 29 of 40 isolates (73%) and to aztreonam (MIC ≥32 μg/ml) in 27 of 40 isolates (67.5%). Among these 40 isolates, 26 (65%), 10 (25%), and 4 (10%) were obtained in urine, sputum, and others, respectively, indicating that urine and sputum may be the most important sources of MDR *P. aeruginosa*. As shown in Table 2, The PER-type β-lactamase gene (bla_{PER}), SHV-type β-lactamase gene (bla_{SHV}), VIM-type β-lactamase gene (bla_{VIM}), IMP-type β-lactamase gene (bla_{IMP}), and OXA-type β-lactamase gene (bla_{OXA}) were detected. Of the 67 isolates tested, 27 isolates (40%) carried the bla_{OXA} gene (class D); 18 (27%) the bla_{VIM} gene (class B); one (1.5%) the bla_{IMP} gene (class B); one (1.5%) the bla_{SHV} gene (class A); and one (1.5%) the bla_{PER} gene (class A). Based on the direct DNA sequencing results, bla_{OXA-10}, bla_{VIM-2}, bla_{OXA-2}, bla_{OXA-17}, bla_{PER-1}, bla_{SHV-12}, and bla_{IMP-1} were carried by 23 (34.3%), 18 (26.9%), 2 (3.0%), 2 (3.0%), 1 (1.5%), 1 (1.5%), and 1 (1.5%) isolates, respectively (Table 2). The most prevalent β-lactamase gene was bla_{OXA-10}, which is consistent with the results of another survey undertaken in Korea [12]. Nineteen isolates resistant to imipenem carried the bla_{VIM-2} and bla_{IMP-1} genes. Four isolates resistant to extended-spectrum cephalosporins (cefotaxime and ceftazidime) carried ESBL genes (bla_{OXA-17}, bla_{PER-1}, and bla_{SHV-12}). Seven (10.4%) isolates contained more than two types of β-lactamase genes: five isolates carried the bla_{VIM-2} and bla_{OXA-10} genes; one isolate (PA5175) carried the bla_{IMP-1} and bla_{OXA-10} genes; and one isolate (PA4106) carried the bla_{PER-1}, bla_{VIM-2}, and bla_{OXA-10} genes. To the best of our knowledge, this is the first report of the coexistence of bla_{OXA-10} with bla_{VIM-2}, bla_{IMP-1}, or bla_{PER-1} in MDR *P. aeruginosa* clinical isolates. Although the complete nucleotide sequences of integrons from these clinical isolates were not determined, screening for the presence of classes 1 through 4 integrase genes by PCR revealed the presence of a class 1 integron (data not shown). The horizontal β-lactamase gene transfer could have occurred by a class 1 integron, In34 [19]. Therefore, the horizontal gene transfer by the class 1 integron can explain why some isolates carry more than one β-lactamase gene. To investigate the complete multidrug resistance mechanisms, further studies of other mechanisms (*e.g.*, decreased outer-membrane permeability, increased efflux, and DNA gyrase mutations) are currently under way. More than one or two β-lactamase genes were carried by 10% of MDR *P. aeruginosa* isolates. In particular, one isolate that contained the bla_{PER-1}, bla_{VIM-2}, and bla_{OXA-10} genes was resistant to all nine antimicrobial agents tested. The emergence of multiple β-lactamase-producing *P. aeruginosa* genes may entail therapeutic failure and failure to eradicate these isolates with any of the antimicrobial agents tested, except aztreonam. Therefore, a more prudent use of these antimicrobial agents is necessary to reduce the spread of these resistant isolates. The nationwide spread of these isolates in Korean community hospitals is extremely clinically significant, because clinicians are advised against the use of these antimicrobial agents.

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