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| 보 문 |

Genotypic Investigation of Multidrug-Resistant *Pseudomonas aeruginosa* from Clinical Isolates in Korea, 2010

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2010년도 국내 임상에서 분리한 다제내성 녹농균의 유전자형 조사

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Pseudomonas aeruginosa* is an opportunistic Gram-negative bacterium that causes serious infection, particularly in immunocompromised patients. Also, *P. aeruginosa* possessing carbapenem-resistant metallo-β-lactamases (MBL) has been reported with increasing frequency in Korea. We therefore analyzed the level of multidrug-resistant clinical *P. aeruginosa* isolated from a secondary hospital in Korea in 2010. A total of 92 isolates of *P. aeruginosa* were collected from Sahmyook Medical Center in 2010. Susceptibility to antimicrobial agents was determined by analysis of the minimum inhibitory concentration test; the inhibitor-potentiated disk diffusion (IPD) test was performed for MBL detection. RAPD-PCR was used for genotyping to rapidly characterize *P. aeruginosa* strains isolated from clinical patients. The percentages of non-susceptible isolates were as follows: 40.2% to ceftazidime, 58.7% to meropenem, 56.5% to gentamicin, 46.7% to tobramycin, 62.0% to ciprofloxacin and 97.8% to chloramphenicol. The 29 multidrug-resistant strains were screened by the IPD test: of the 21 PCR-positive isolates, 19 were IPM-1 producers and 2 were VIM-2 producers. Among the 19 IMP-1-producing *P. aeruginosa* isolates, 16 isolates showed similar patterns, and three different banding patterns were observed. The proportion of IMP-1-producing multidrug-resistant *P. aeruginosa* from clinical isolates steadily increased in this secondary hospital in Korea in 2010. This study provides information about the antimicrobial-resistant patterns and genotype of multidrug-resistant *P. aeruginosa* isolated from clinical isolates in Korea, 2010.

***Keywords*:** *Pseudomonas aeruginosa*, antimicrobial resistance, metallo-β-lactamases (MBL), multidrug-resistant

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium that causes serious infection, particularly in immunocompromised patients. They play an important role in hospital intensive care units where they cause a wide spectrum of nosocomial infections (Fernández-Cuenca *et al*., 2003). Moreover, nosocomial *P. aeruginosa* strains exhibit high rates of resistance to antibiotics and are frequently multidrug-resistant. Increasing resistance to many antibiotics has been observed in *P. aeruginosa* and presents a major therapeutic dilemma (Anna *et al*., 1999; Landman and Quale, 2002; Livermore, 2002).

Carbapenem antibiotics such as imipenem and meropenem are last-resort drugs of choice to treat serious infections caused by multidrug-resistant *P. aeruginosa.* Development of antibiotic resistance to most classes of antibiotics, including carbapenems, has made it difficult to treat such infections even with combination therapy. Intensive use of carbapenems in the treatment of nosocomial *P. aeruginosa* infections has facilitated the emergence of mechanisms that confer resistance to carbapenems, such as impermeability of the outer membrane, production of carbapenemases, the overexpression of efflux pumps and alterations in penicillin-binding proteins (PBPs) (Tam *et al*.,

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Table 1. Prevalence of *P. aeruginosa* isolated from clinical samples

Characteristics	Total N (%)	Male N (%)	Female N (%)
Number	92	48 (52.2)	44 (47.8)
Age			
<60	27 (29.3)	14 (15.2)	13 (14.1)
≥60	65 (70.7)	34 (37.0)	31 (33.7)
Specimens			
Sputum	59 (64.1)	33 (35.8)	26 (28.3)
Urine	30 (32.6)	14 (15.2)	16 (17.4)
Stool	1 (1.1)	1 (1.1)	ND[a]
Bronchial wash	1 (1.1)	ND[a]	1 (1.1)
Bile	1 (1.1)	ND[a]	1 (1.1)

[a] ND: Not detected.

2007). The most important carbapenemases in *P. aeruginosa* are class B metallo-β-lactamases (MBL) such as VIM and IMP types (Zhiyong *et al*., 2003). Twenty-five IMP variants have been described; IMP-1 was the first MBL identified in *P. aeruginosa* and its national spread in Gram-negative bacilli was reported in Japan (Senda *et al*., 1996). Among the VIM types, VIM-2 is the most dominant worldwide and was particularly detected in Europe (Livermore and Woodford, 2000). Furthermore, carbapenem-resistant MBL-possessing *P. aeruginosa* has been reported with increasing frequency in Korea (Oh *et al*., 2003; Lee *et al*., 2009; Kim *et al*., 2011).

In this study, we analyzed the levels of multidrug-resistant clinical *P. aeruginosa* isolated from a secondary hospital in Korea in 2010. Six antibiotics that are widely used in clinical patients were selected. In addition, epidemiological studies were performed using RAPD-PCR to define the genetic similarity between VIM and IMP types from isolates.

Materials and Methods

Clinical isolates

A total of 92 non-repetitive isolates of *P. aeruginosa* were collected from Sahmyook Medical Center in 2010. *P. aeruginosa* was recovered from various clinical specimens: sputum (64.1%), urine (32.6%), stool (1.1%), bronchial wash (1.1%) and bile (1.1%), and stored at -70℃ in nutrient broth containing 80% glycerol.

Susceptibility test

The 92 isolates were grown overnight on Mueller Hinton broth (Difco) at 37℃ for 24 h, and were then tested for resistance to the six antimicrobial agents. Minimal inhibitory concentrations (MIC) of the various agents were determined by the agar dilution method, according to the guidelines established by the Clinical Laboratory Standards Institute (CLSI, 2007). *P. aeruginosa* ATCC 27853 was concurrently tested as a quality control strain. The antibiotics tested were ceftazidime (GlaxoSmithKline), gentamicin (Sigma), ciprofloxacin (Ildong), chloramphenicol (Fluka AG), meropenem (Yuhan) and tobramycin (Daewoong). Results were included in the analysis only when the quality control isolate results were within the acceptable range according to CLSI guidelines. We selected 29 multidrug resistant isolates from the original 92 isolates. The resistant isolates were identified by Samkwang Medical Laboratories.

Metallo-*β*-lactamase (MBL) screening

The inhibitor-potentiated disk diffusion (IPD) test was performed for the detection of MBL producing clinical isolates of multidrug *P. aeruginosa* (Yong *et al*., 2002). The surface of a Muller-Hinton agar plate was inoculated with a culture suspension of the test strain as recommended by the Clinical Laboratory Standards Institute. After drying, two 10-μg imipenem disks (Becton Dickinson) were placed on the plate at a 3–4 cm center-to-center distance. Subsequently, 10 μl of 0.5 M EDTA (Seoul chemical) solution was added to one of them. After incubation for 18 h, a difference in the inhibition zones of the imipenem and imipenem-EDTA disks of more than 7 mm was interpreted as a positive IPD test.

MBL gene detection

The bla_{IMP-1} and bla_{VIM-2} genes were detected by PCR experiments with the following primers: IMP-F (5′-CAT GGT TTG GTG GTT CTT GT-3′), IMP-R (5′-ATA ATT TGG CGG ACT TTG GC-3′) and VIM-F (5′-ATT GGT CTA TTT GAC CGC GTC-3′), VIM-R (5′-TGC TAC TCA ACG ACT GAG CG-3′). All amplifications were carried out according to the corresponding conditions (Jeon *et al*., 2005).

RAPD analyses

Table 2. Antimicrobial susceptibility patterns of *P. aeruginosa* clinical isolates

Antimicrobial agent	MIC (μg/ml)		Sensitive N (%)	Intermediate N (%)	Resistance N (%)
	50%	90%			
Ceftazidime	4	128	55 (59.8)	2 (2.2)	35 (38.0)
Meropenem	16	>128	38 (41.3)	2 (2.2)	52 (56.5)
Gentamicin	8	>128	40 (43.5)	4 (4.3)	48 (52.2)
Tobramycin	1	>128	49 (53.3)	0 (0.0)	43 (46.7)
Ciprofloxacin	16	>64	35 (38.0)	0 (0.0)	57 (62.0)
Chloramphenicol	128	>128	2 (2.2)	1 (1.1)	89 (96.7)

Clonal relatedness of the isolates was assessed by RAPD-PCR. Genomic DNA was extracted with the QIAamp DNA mini kit (Qiagen), according to the manufacturer's instructions. All amplified PCR products were resolved by electrophoresis in 2% agarose gel and stained with SYBR safe DNA gel stain (Gibco). PCR reactions were carried out in 50 μl reaction mixtures containing the DNA template, 20 mM Tris-HCl, 100 mM KCl, 25 mM $MgCl_2$, 2.5 mM of each dNTP, primer, and 5 units of *Taq* DNA polymerase (TaKaRa, Japan). The experiments were done with BOXA1R (5′-CTA CGG CAA GGC GAC GCT GAC G-3′) with the following amplification conditions: initial denaturation at 92℃ for 2 min; followed by 35 cycles consisting of 30 sec at 92℃, 1 min at 52℃ and 2 min at 72℃; and a final cycle of 72℃ for 5 min, after which the PCR products were chilled to 4℃ (Koeuth *et al.*, 1995). PCR products were visualized under UV light (VILBER Lourmat, France) at 254 nm. The amplification reaction was repeated three times to establish reproducibility.

Results and Discussion

Antimicrobial therapy is limited against infections caused by multidrug-resistant *P. aeruginosa.* Many studies have reported significant relationships between antibiotic consumption and resistance, because inappropriate use of antibiotic has created a serious problem in the choice of therapy.

Susceptibility test

Among 92 patients infected with *P. aeruginosa*, 48 (52.2%) were male and 44 (47.8%) were female (Table 1). The majority of *P. aeruginosa* were isolated from sputum (64.1%) and urine (32.6%). The percentages of non-susceptible isolates were as follows: 40.2% to ceftazidime, 58.7% to meropenem, 56.5% to gentamicin, 46.7% to tobramycin, 62.0% to ciprofloxacin and 97.8% to chloramphenicol (Table 2). The most active antibiotic was ceftazidime (resistance in 38.0%), and the least active antimicrobial agent was chloramphenicol (resistance in 96.7%).

β-Lactamase genotyping

In the present study, 29 (31.5%) multidrug-resistant (MDR, resistant to three or more antimicrobial classes) isolates were identified among 92 clinical *P. aeruginosa* isolates collected from a secondary hospital in Korea in 2010. The 29 MDR strains were mostly found in urine specimens (75.8%), indicating that urine may serve as an important reservoir for dissemination of resistant bacteria. The average patient age was 67.2 and 54.4 for males and females, respectively; most multidrug-resistant bacteria were derived from elderly patients around 60 years of age (58.6%).

The 29 multidrug-resistant strains were screened by IPD test; 21 were positive for MBL and 8 were negative in this screening test. Of the 21 PCR-positive isolates, 19 were IPM-1 producers and 2 were VIM-2 producers (Table 3). Nucleotide sequencing revealed that all the bla_{IMP-1} and bla_{VIM-2} were IMP-1 and VIM-2, respectively. In this study, IPD test results showed that 21 out of 29 isolates produced MBL, but genotyping PCR was not successful in 8 strains. It is possible to obtain a false-positive by using EDTA as MBL inhibitor (Yong, 2009), which suggests that other MBL genes are involved in genotype generation. Based on the determined mechanism of resistance, further studies are required.

Two major MBL families, IMP and VIM, have been found in gram negative bacteria, especially in the Asian Pacific region and Latin America. They have low amino acid homology (approximately 30% to 40%), but their hydrolytic properties are similar (Gibb *et al.*, 2002). Likewise, both are often encoded by mobile gene cassettes inserted into integrons that are sometimes located on plasmids (Levasque *et al.*, 1995; Bush, 1998). Several close relatives of IMP-1 have been reported: IMP-2 from an isolate of *A. baumannii* in Italy (Riccio *et al.*, 2000), IMP-3 from *Shigella flexneri* in Japan (Iyobe *et al.*, 2000), and IMP-4 from *Acinetobacter* and *Citrobacter* spp. from Hong Kong (Chu *et al.*, 2001). VIM-2 which shares 90% amino acid identity with VIM-1. To date, eleven VIM variants have been reported worldwide, mostly in European and Asian countries (Walsh *et al.*, 2005). In comparison with domestic

Table 3. Antibiotic susceptibility patterns and β-lactamases of multidrug-resistant *P. aeruginosa*

Test No.	Isolates No.	Antibiotic susceptibilities						β-Lactamase type
		CHL	TOB	GEN	MEM	CAZ	CIP	
1	PA3	R	R	R	R	R	R	IMP-1
2	PA5	R	R	R	R	R	R	ND[a]
3	PA7	R	R	R	R	R	R	VIM-2
4	PA8	R	R	R	R	R	R	ND[a]
5	PA11	R	R	R	R	R	R	VIM-2
6	PA19	R	R	R	R	R	R	ND[a]
7	PA20	R	R	R	R	R	R	ND[a]
8	PA21	R	R	R	R	R	R	IMP-1
9	PA22	R	R	R	R	R	R	IMP-1
10	PA23	R	R	R	R	R	R	IMP-1
11	PA24	R	R	R	R	R	R	IMP-1
12	PA25	R	R	R	R	R	R	IMP-1
13	PA26	R	R	R	R	R	R	IMP-1
14	PA27	R	R	R	R	R	R	IMP-1
15	PA28	R	R	R	R	R	R	IMP-1
16	PA29	R	R	R	R	R	R	IMP-1
17	PA46	R	R	R	R	R	R	ND[a]
18	PA47	R	R	R	R	R	R	IMP-1
19	PA48	R	R	R	R	R	R	IMP-1
20	PA49	R	R	R	R	R	R	IMP-1
21	PA59	R	R	I	R	R	R	IMP-1
22	PA74	R	R	I	I	S	R	ND[a]
23	PA77	R	R	R	R	R	R	ND[a]
24	PA78	R	R	R	R	R	R	ND[a]
25	PA80	R	R	R	R	R	R	IMP-1
26	PA81	R	R	R	R	R	R	IMP-1
27	PA84	R	R	R	R	R	R	IMP-1
28	PA85	R	R	R	R	I	R	IMP-1
29	PA87	R	R	R	R	R	R	IMP-1

CHL, Chloramphenicol; TOB, Tobramycin; GEN, Gentamicin; MEM, meropenem; CAZ, Ceftazidime; CIP: Ciprofloxacin; I, Intermediate; R, Resistant; S, Susceptible
[a] ND, Not detected.

studies in past years (Lee *et al*., 2002; Kim *et al*., 2004), we found IMP-1 in 65.5% of isolates and VIM-2 in 6.9%. The proportion of bla_{IMP}-positive isolates was greater than that of bla_{VIM}-positive isolates. Also IMP-1-producing *P. aeruginosa* isolates were resistant to all antibiotics except meropenem and ceftazidime. Based on recent reports, our results also indicate that the proportion of IMP-1-producing *P. aeruginosa* is growing steadily (Kim *et al*., 2002; Lee *et al*., 2004).

RAPD analyses

Among the 19 IMP-1 producing *P. aeruginosa* isolates, 16 isolates showed similar patterns, and three different banding patterns were observed. The two VIM-2-producing *P. aeruginosa* isolates showed related patterns. These isolates had two common bands of approximately 800 bp and 500 bp (Fig. 1). The RAPD-PCR results suggest that most of the MBL-producing *P. aeruginosa* isolates were genetically related. But, the three different band patterns suggest a horizontal spread in this species.

In conclusion, the proportion of IMP-1-producing multidrug-resistant *P. aeruginosa* from clinical isolates steadily increased in secondary hospitals in Korea in 2010. This study provides information about the antimicrobial-resistant patterns and genotype of multidrug-resistant *P. aeruginosa* isolated from clinical isolates in Korea, 2010. Antibiotic use and treatment should be restricted as part of a strategy to control multidrug-resistant *P. aeruginosa.*

적 요

녹농균은 특히 면역이 저하된 환자에게서 심각한 감염을 일으키는 그람음성의 기회감염 균주이다. 또한 carbapenem 내성

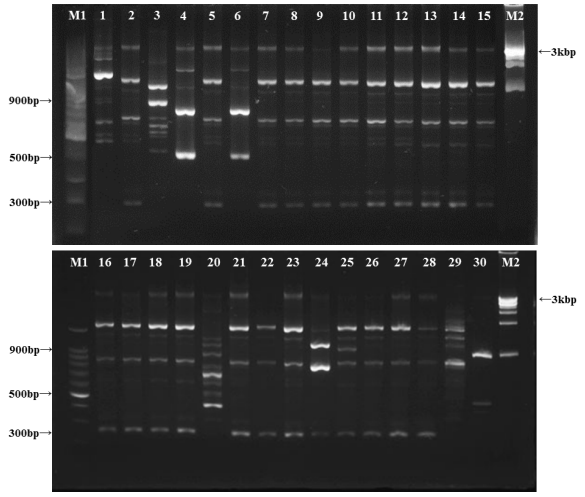

Fig. 1. BOX-PCR patterns of genomic DNA from clinical isolates of multidrug-resistant *P. aeruginosa*. Lanes: M1, 100 bp; M2, 1-kb DNA size marker; 1, *P. aeruginosa* ATCC27853; 2, PA3; 3, PA5; 4, PA7; 5, PA8; 6, PA11; 7, PA19; 8, PA20; 9, PA21; 10, PA22; 11, PA23; 12, PA24; 13, PA25; 14, PA26; 15, PA27; 16, PA28; 17, PA29; 18, PA46; 19, PA47; 20, PA48; 21, PA49; 22, PA59; 23, PA74; 24, PA77; 25, PA78; 26, PA80; 27, PA81; 28, PA84; 29, PA85; 30, PA87.

metallo-β-lactamases (MBL)를 가진 녹농균이 한국에서 증가되는 추세로 보고되고 있다. 따라서 본 실험에서는 2차 병원인 삼육 서울 병원에서 수집된 총 92종의 임상 녹농균의 다재내성 수준을 분석하였다. 항생제에 대한 감수성은 최소억제농도(MIC) 분석에 의해 결정되었고, inhibitor-potentiated disk diffusion (IPD) 분석은 MBL 검출을 위해 수행되었다. RAPD-PCR은 임상환자에서 분리한 녹농균 계통의 유전적 유형의 특징을 밝히기 위해 사용되었다. 그 결과 임상에서 분리된 녹농균의 40.2%는 ceftazidime에 내성을, 58.7%는 meropenem에 내성을, 56.5%는 gentamicin에 내성을, 46.7%는 tobramycin에 내성을, 62.0%는 ciprofloxacin에 내성을 그리고 97.8%는 chloramphenicol에 내성을 보였다. IPD 분석에 의해 29종의 다재내성 균주로 관찰 되었고, RAPD 분석에 의해 19종은 IPM-1 유전자형을, 2종은 VIM-2 유전자형을 만들었다. MBL 유전자 검출 시험을 통해 19종의 IMP-1 생성 녹농균 중에서 16종이 유사한 유전자형을 보였고, 3종은 다른 유전자형이 관찰되었다. 임상에서 분리한 IMP-1 생성 다재내성 녹농균의 비율은 꾸준히 증가하고 있다. 이번 연구는 2010년 국내 임상에서 분리한 녹농균의 항생제 다제내성 패턴과 유전자형에 대한 정보를 제공한다.

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