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First Detection of bla_{IMP-1} in Clinical Isolate Multiresistant *Acinetobacter baumannii* from Korea

JEONG SEOK HOON[1], IL KWON BAE[1], SEUNG GHYU SOHN[2], KWANG OK PARK[1], YOUNG JUN AN[2], KWANG HOON SUNG[2], SEON JU JANG[2], MYONG JIN HEO[2], KI SUK YANG[2], AND SANG HEE LEE[2]*

[1]*Department of Laboratory Medicine, College of Medicine, Kosin University, Busan 602-702, Korea*
[2]*Department of Biological Sciences, School of Biotechnology and Environmental Engineering, Myongji University, Yongin 449-728, Korea*

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Abstract Among 46 *Acinetobacter baumannii* isolates collected in 2004, two imipenem-resistant isolates were obtained from clinical specimens taken from patients hospitalized in Busan, Republic of Korea. Two carbapenemase-producing isolates were further investigated to determine the mechanism of resistance. These isolates were analyzed by antibiotic susceptibility testing, microbiological tests of carbapenemase activity, determination of pI, transconjugation test, enterobacterial repetitive consensus (ERIC)-PCR, and DNA sequencing. Two cases of infection by *A. baumannii* producing the IMP-1 β-lactamase were detected. The isolates were characterized by a modified cloverleaf synergy test and EDTA-disk synergy test. Isoelectric focusing of crude bacterial extracts revealed nitrocefin-positive bands with a pI value of 9.0. PCR amplification and characterization of the amplicons by direct sequencing indicated that the isolates carried a bla_{IMP-1} determinant. The isolates were characterized by a multidrug resistance phenotype, including penicillins, extended-spectrum cephalosporins, carbapenems, and aminoglycosides. These results indicate that the observed imipenem resistance of two Korean *A. baumannii* isolates was due to the spread of an IMP-1-producing clone. Our microbiological test of carbapenemase activity is simple to screen class B metallo-β-lactamase-producing clinical isolates to determine their clinical impact and to prevent further spread. This study shows that the bla_{IMP-1} resistance determinant, which is emerging in Korea, may become an emerging therapeutic problem, since clinicians are advised not to use extended-spectrum cephalosporins, imipenem, and aminoglycosides. This observation emphasizes the importance of having effective control measures in Asian hospitals, such as early detection of colonized patients, isolation procedures, and a judicious use of antibiotics.

*Corresponding author
Phone: 82-31-330-6195; Fax: 82-31-335-8249;
E-mail: sangheelee@mju.ac.kr

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Acinetobacter baumannii has emerged as an important nosocomial pathogen in outbreaks of hospital infections and ranks second to *Pseudomonas aeruginosa* among nosocomial pathogens of aerobic nonfermentative Gram-negative bacilli [7, 15, 28, 31]. *A. baumannii* causes respiratory and urinary tract infections, meningitis, endocarditis, burn infections, and wound sepsis, especially in intensive care units (ICUs) [6]. *A. baumannii* infections are often difficult to eradicate because of high-level resistance to many antibiotics due to both intrinsic and acquired mechanisms. β-Lactamase production is the most important mechanism of acquired β-lactam resistance in Gram-negative pathogens [33]. Carbapenems (*e.g.*, imipenem and meropenem) have become the drugs of choice against *Acinetobacter* infections in many medical centers, but are being compromised by the emergence of carbapenem-hydrolyzing β-lactamase (carbapenemase) of molecular classes B and D [17, 21]. Class B carbapenemases found thus far in *Acinetobacter* spp. include various IMP- and VIM-type metallo-β-lactamases (http://www.lahey.org/studies/webt.asp), and *Acinetobacter* spp. also produce zinc-independent members of molecular class D OXA-type β-lactamases [1–4, 9, 10, 25]. Recently, new subclasses of metallo-β-lactamases such as SPM-1 and GIM-1 have been reported [5].

Forty-six *A. baumannii* isolates were collected from 46 patients hospitalized at the Kosin University Gospel Hospital, from June to September 2004. The purpose of the present study was to investigate the prevalence of imipenem-resistant *A. baumannii* in the Republic of Korea and to characterize the imipenem resistance mechanism of the isolates.

MATERIALS AND METHODS

Bacterial Strains

A total of 46 nonrepetitive clinical isolates of *A. baumannii* were collected from June to September 2004 at the Kosin University Gospel Hospital (Busan, Republic of Korea), which has 1,300 patient beds. These isolates were collected from different patients hospitalized at the intensive care unit (ICU), and at the general, neurology, and urology wards. The isolates were identified by conventional techniques [28] and/or Vitek GNI card (bioMérieux Vitek Inc., Hazelwood, MO, U.S.A.). *A. baumannii* YMC02/8/P535 [33] was used as the recipient strain for transfer by transconjugation. *Escherichia coli* ATCC 25922 was used as the MIC reference strain.

Susceptibility of β-Lactams

Antibiotic susceptibility was determined by disk diffusion tests that were performed according to the manufacturer's instructions with BBL (Cockeysville, MD, U.S.A.) disks impregnated with amikacin (30 μg), gentamicin (10 μg), tobramycin (10 μg), tetracycline (30 μg), trimethoprim-sulfamethoxazole (1.25 and 23.75 μg, respectively), ciprofloxacin (5 μg), ampicillin (10 μg), ampicillin-sulbactam (10 and 10 μg, respectively), piperacillin (100 μg), piperacillin-tazobactam (100 and 10 μg, respectively), cefoxitin (30 μg), cefotetan (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), cefepime (30 μg), aztreonam (30 μg), imipenem (10 μg), and meropenem (10 μg). Disks were dispensed with a BBL Sensi-Disc 12-place dispenser. MICs were determined by the agar dilution technique on Muller-Hinton agar plates (Becton-Dickinson, Sparks, MD, U.S.A.) containing serially two fold-diluted β-lactams as described previously [23].

Microbiological Tests of Carbapenemase Activity

In order to study the inactivation of imipenem by the *A. baumannii* class B and/or D β-lactamases, a microbiological disk synergy test was performed. The cloverleaf test of Hornstein *et al.* [11] was modified by substituting *E. coli* ATCC 25922 for imipenem-susceptible *Micrococcus luteus*. The surface of a Muller-Hinton agar plate was evenly inoculated using a cotton swap with an overnight culture suspension of *E. coli*, which was adjusted to the turbidity of the McFarland no. 0.5 tube. After brief drying, an imipenem disk (30 μg) was placed at the center of the plate, and imipenem-resistant test strains from the overnight culture plates were streaked from the edge of the disk to the periphery of the plate. The presence of a distorted inhibition zone after overnight incubation was interpreted as a positive modified cloverleaf synergy test, showing the inactivation of imipenem by carbapenemase (class B and/or D).

Microbiological testing of metallo-β-lactamase activity was performed by an EDTA-disk synergy test [18] modified as follows: An overnight culture of the test strain was suspended to the turbidity of the McFarland no. 0.5 tube and used to swab the inoculate on a Muller-Hinton agar plate. After drying, a 30-μg imipenem disk (BBL) and a blank filter paper disk were placed 15 mm apart from edge to edge, and 10 μl of 0.5 M EDTA solution was then applied to the blank disk, which resulted in about 1.5 mg/disk. After overnight incubation, the presence of enlarged zone of inhibition was interpreted as a positive EDTA-disk synergy test, showing the inactivation of metallo-β-lactamase (class B) activity by EDTA.

Plasmid Preparation and Southern Blot Analysis

Isolation of plasmid DNA from *A. baumannii* clinical isolates was performed as described by Sambrook and Russel [27] with plasmid-safe ATP-dependent DNase (Epicentre Technology, Madison, WI, U.S.A.) to remove contaminated bacterial chromosomal DNA. The prepared plasmids were separated in 1.0% agarose using a field inversion gel electrophoresis (FIGE) Mapper Electrophoresis System (Bio-Rad, Hercules, CA, U.S.A.).

Transfer of DNA to a nylon membrane (Hybond-N; Amersham International, Buckinghamshire, England) was performed essentially as described by Sambrook and Russel [27]. Labeling of DNA (PCR products for bla_{IMP} gene) probe was performed with digoxigenin as described by the manufacturer (Boehringer Mannheim Biochemicals, IN, U.S.A.). Hybridization was performed at 68°C with buffers recommended in the instructions included in the digoxigenin kit purchased from Boehringer Mannheim Biochemicals.

Transconjugation Experiments

Curing was attempted by growing cultures overnight in nutrient broth containing ethidium bromide (Sigma-Aldrich, Louis, MO, U.S.A.) at 0.25–0.5 times the MIC, followed by replica plating onto Muller-Hinton agar plates with and without imipenem at 2 μg/ml or 10 μg/ml. Transconjugation experiments were performed as described previously [24] with a rifampin-resistant *A. baumannii* YMC02/8/P535 as recipient. Transconjugants were selected on Muller-Hinton agar supplemented with rifampin (Sigma-Aldrich) (100 μg/ml) to inhibit the growth of the donor strain and with imipenem (1 μg/ml) to inhibit the growth of the recipient strain.

Isoelectrofocusing

Crude bacterial extracts were obtained from *A. baumannii* clinical isolates after centrifugation of sonicated culture, as previously described [19]. Sonic extracts were used for the determination of isoelectric points (pIs) and β-lactamase activity. Isoelectrofocusing (IEF) was performed in Ready Gel precast IEF polyacrylamide gels (Bio-Rad) as previously described [19]. Gels were developed with 0.5 mM nitrocefin (Merck, Whitehouse Station, NJ, U.S.A.).

Table 1. Nucleotide sequences of oligonucleotides used for PCR amplifications and sequencing of bla_{IMP}-, bla_{VIM}-, bla_{GIM}-, and bla_{SPM}-type genes.

Primer	Sequence (5'→3')	Target β-lactamase	Accession no. of *bla*[b]
IMP-1F[a]	GCTACCGCAGCAGAGTCTTTG	IMP-1, IMP-3, IMP-6, IMP-10	S71932, AB010417, AB040994,
IMP-1R[a]	CCTTTAACCGCCTGCTCTAATG		AY074433
IMP-2F	ATGTTACGCAGCAGGGCAG	IMP-2, IMP-8, IMP-10, IMP-12, IMP-13	AJ243491, AF322577, AY074433,
IMP-2R	ATGCTCAGTCATGAGGCGC		AJ420864, AJ550807
IMP-4F	GAAGGCGTTTATGTTCATACTTCG	IMP-4, IMP-5, IMP-7, IMP-9	AF244145, AF290912, AF318077,
IMP-4R	GCGTCACCCAAATTACCTAGACC		AY033653
IMP-11F	GAGAAGCTTGAAGAGGGTGTTTAT	IMP-11, IMP-12, IMP-21	AB074436, AJ420864, AB204557
IMP-11R	AGGTAGCCAAACCACTACGTTATC		
IMP-18F	CATTGCTGCTGCAGATGATTC	IMP-18	AY780674
IMP-18R	CTGCAAGAGTGATGCGTTTC		
IMP-19F	GTTTTATGTGTATGCTTCCTTTGTAGC	IMP-19, IMP-20	AB201265, AB196988
IMP-19R	CAGCCTGTTCCCATGTACG		
VIM-1F	GTTTGGTCGCATATCGCAAC	VIM-1, VIM-4, VIM-5, VIM-7, VIM-11	Y18050, AY135661, AY144612,
VIM-1R	AGACCGCCCGGTAGACC		AJ536835, AY635904
VIM-2F	GTTTGGTCGCATATCGCAAC	VIM-2, VIM-3, VIM-6, VIM-8,	AF191564, AF300454, AY165025,
VIM-2R	CTACTCAACGACTGAGCGATTTGT	VIM-9, VIM-10	AY524987, AY524988, AY524989
GIM-1F	CAGGGTCATAAACCGCTAGAAG	GIM-1	AJ620678
GIM-1R	AACTTCCAACTTTGCCATGC		
SPM-1F	GAGAGCCCTGCTTGGATTC	SPM-1	AY341249
SPM-1R	GCGACCTTGATCGTCTTGTT		

[a]Orientation of each primer: F, forward; R, reverse.

[b]β-Lactamase genes (*bla*) used in the multiple sequence alignment for designing each primer pair.

PCR Amplification and DNA Sequencing

Unless otherwise stated, molecular biological reagents and restriction enzymes were obtained from Sigma-Aldrich. Genomic DNA of clinical isolates were prepared with a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, U.S.A.) and used as template DNA in PCR amplification. The primers for PCR amplification were designed by selecting consensus sequences in multiple-nucleotide alignment of the IMP-type β-lactamase genes (bla_{IMP}), VIM-type β-lactamase genes (bla_{VIM}), GIM-1 β-lactamase gene (bla_{GIM-1}), and SPM-1 β-lactamase gene (bla_{SPM-1}), by using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers are described in Table 1. PCR amplifications were carried out as described previously [13, 14, 19, 20, 29].

DNA sequencing was performed by the direct sequencing method with an automatic sequencer (ABI PRISM3100; Applied Biosystems, Weiterstadt, Germany), as previously described [20]. DNA sequence analysis was performed with DNASIS for Windows (Hitachi Software Engineering America Ltd., San Bruno, CA, U.S.A.). Database similarity searches for both the nucleotide sequences and deduced protein sequences were performed with BLAST at the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov).

Enterobacterial Repetitive Consensus PCR

Enterobacterial repetitive consensus (ERIC)-PCRs were performed in 50-μl volumes containing 10 ng of genomic DNA from *A. baumannii* clinical isolates, 4 mM $MgCl_2$, 50 pM each of primer, ERIC1R (5'-ATGTAAGCTCCTG-GGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACT-GGGGTGAGCG-3') [32], 1.25 U of TaKaRa Ex *Taq* polymerase (TaKaRa, Otsa, Shiga, Japan), 0.2 mM each of dATP, dCTP, dGTP, and dTTP in 25 mM TAPS [*N*-Tris(hydroxy)methyl-3-amino-propane sulfonic acid, pH 9.3], 50 mM KCl, and 1 mM 2-mercaptoethanol. Amplification was carried out using the following program: 95°C for 5 min followed by 35 cycles of 1 min at 52°C, 5 min at 70°C, and 1 min at 92°C. The final extension step was performed at 70°C for 10 min. The analysis of amplified products (10-μl aliquots) was performed in 2% Seakem LE agarose (BMA, Rockland, ME, U.S.A.). For pulsed-field gel electrophoresis (PFGE), SmaI-digested genomic DNA was prepared according to the instruction of Bio-Rad (Hercules, CA, U.S.A.), and fragments were separated for 20 h at 6 V/cm at 11°C using a CHEF-DRII system (Bio-Rad), with initial and final pulse times of 0.5 and 60 s, respectively. DNA fingerprints were interpreted as recommended by Zarrilli *et al.* [34].

RESULTS

Phenotypic Properties of Imipenem-Resistant Isolates

Twenty-seven (59%), thirteen (28%), four (9%), and two (4%) among 46 nonrepetitive *A. baumannii* isolates were

Table 2. Antimicrobial susceptibility of *A. baumannii* isolates.

Antimicrobial agent	Resistance (%)[a]		
	IMP-S (n=44)	IMP-R (n=2)	Total (n=46)
Ampicillin	93.2	100	93.5
Ampicillin-sulbactam	0	100	4.3
Piperacillin	2.3	100	4.3
Piperacillin-tazobactam	2.3	100	4.3
Cefoxitin	86.4	100	87.0
Cefotetan	97.9	100	97.8
Ceftazidime	4.5	100	6.5
Cefotaxime	13.6	100	17.4
Cefepime	2.3	100	4.3
Aztreonam	63.6	50	63.0
Imipenem	0	100	4.3
Meropenem	0	100	4.3
Amikacin	6.8	100	10.9
Gentamicin	9.1	100	13.0
Tobramycin	9.1	100	13.0
Tetracycline	6.8	50	8.7
Trimethoprim-sulfamethoxazole	11.4	50	13.0
Ciprofloxacin	4.5	50	6.5

[a]IMP-S, imipenem-susceptible; IMP-R, imipenem-resistant.

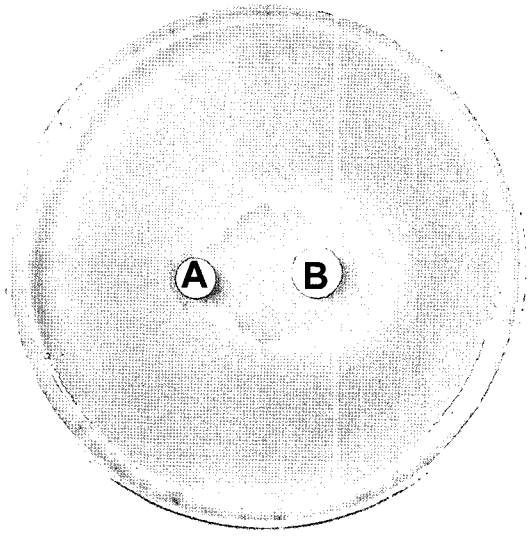

Fig. 1. EDTA-disk synergy test for a representative IMP-1-producing isolate (*A. baumannii* AB420673).
An overnight culture of the test strain AB420673 was suspended to the turbidity of the McFarland no. 0.5 tube and used to swab the inoculate on a Muller-Hinton agar plate. After drying, a 30-μg imipenem disk (**A**) and a blank filter paper disk (**B**) were placed 15 mm apart from edge to edge, and 10 μl of 0.5 M EDTA solution was then applied to the blank disk, which resulted in about 1.5 mg/disk. After overnight incubation, the presence of enlarged zone of inhibition was interpreted as a positive EDTA-disk synergy test, showing the inactivation of metallo-β-lactamase (class B) activity by EDTA.

recovered from the general ward, ICU, urology ward, and neurology ward, respectively. Twenty-three (50%), eighteen (39%), three (7%), and two (4%) among 46 isolates were obtained in sputum, urine, pus, and blood, respectively. This result indicates that sputum and urine may be more important sources of spread than pus and blood. The results of antimicrobial susceptibility test of 46 isolates showed that the prevalence of resistance to ampicillin, cefoxitin, cefotetan, and aztreonam were high (93.5, 87.0, 97.8, and 63.0%, respectively). The prevalence of isolates resistant to all other antimicrobial agents tested was less than 17.4% (Table 2). There was a low detection (4.3%, 2 of 46) of imipenem-resistant isolates. In Korea, the incidence of imipenem resistance among *A. baumannii* from June to September 2004 were lower than the incidence (26.9%) in 2003, which may be due to the reduced prescription of imipenem and a judicious use of imipenem [12]. A total of two isolates of imipenem-resistant *A. baumannii* were studied by the modified cloverleaf synergy test and the EDTA-disk synergy test. Metallo-β-lactamase-producing isolates (positive EDTA-disk synergy test) were detected in two isolates (Fig. 1 and Table 3). The prevalence of carbapenemase-producing isolates (modified cloverleaf synergy test; positive) was 100% (Table 3). These results were confirmed with the carbapenemase activity data by spectrophotometric assays in the presence and absence of EDTA. These results suggest that two imipenem-resistant *A. baumannii* isolates do produce class B metallo-β-lactamases. Two β-lactamase-producing *A. baumannii*

Table 3. Characterization of imipenem-resistant *A. baumannii* isolates.

Isolate	Age/Sex	Type of specimen	Ward[c]	MICs (μg/ml) of β-lactams[c]									β-Lactamase	Synergy test	
				AMP	SAM	PIP	TZP	CAZ	CAZ-CLA	FEP	IMP	MEM		Cloverleaf	EDTA-disk
AB420673	71/M	Urine	GW	>256	16	>256	>256	>256	>256	128	32	32	IMP-1	+	+
AB421793	46/M	Urine	GW	>256	16	>256	>256	>256	>256	256	32	64	IMP-1	+	+
AB420039[a]	58/F	Sputum	GW	>256	16	>256	64	>256	>256	>256	1	1	ND[b]	-	-

[a]Imipenem-susceptible strain.

[b]ND, not detectable for carbapenemase.

[c]Abbreviation: AMP, ampicillin; SAM, ampicillin-sulbactam (2:1 ratio of β-lactam to inhibitor); PIP, piperacillin; TZP, piperacillin-tazobactam (inhibitor fixed at 4 μg/ml); CAZ, ceftazidime; CAZ-CLA, ceftazidime-clavulanic acid (inhibitor fixed at 4 μg/ml); FEP, cefepime; IMP, imipenem; MEM, meropenem; GW, general ward.

showed high levels of resistance to ampicillin, piperacillin, piperacillin-tazobactam, ceftazidime, ceftazidime-clavulanic acid, cefepime, imipenem, and meropenem (Table 3). All two β-lactamase-producing *A. baumannii* isolates were only intermediate to ampicillin-sulbactam of the antibiotics tested. Two isolates produced a β-lactamase with an apparent pI of 9.0 (data not shown). Based on IEF results and previous reports of IMP-1-producing *P. aeruginosa* [16, 26], we suspected that the IMP-1 (class B) carbapenemase could be involved in imipenem resistance.

Molecular Characterization of IMP-1-Producing *A. baumannii* Isolates

Neither transfer nor curing of imipenem resistance was achieved for any imipenem-resistant isolate, despite multiple attempts. bla_{IMP-1}-carrying plasmid DNA was apparently not detectable either in plasmid preparation without contaminated bacterial chromosomal DNA by plasmid-safe ATP-dependent DNase or in whole genomic DNA preparations from two isolates. In a Southern blot experiment carried out with genomic DNA of two isolates, the bla_{IMP-1} probe was hybridized to the band of chromosomal DNA. Two clinical isolates gave PCR products with primer pairs for the bla_{IMP-1} gene, but not with other primer pairs for bla_{VIM}, bla_{GIM}, and bla_{SPM}. Taking into account the resistance phenotypes of two clinical isolates, the resistance genotypes of these isolates were analyzed by direct sequencing of the PCR-amplified fragments specific for the bla_{IMP-1} gene. On the basis of DNA sequencing, two clinical isolates were determined to harbor the bla_{IMP-1} gene. The IMP-1

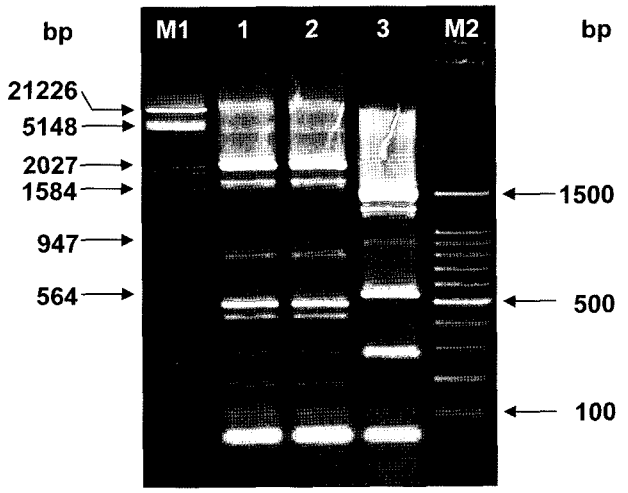

Fig. 2. ERIC-PCR patterns of genomic DNA from three *A. baumannii* clinical isolates: imipenem-resistant (IMP-1-producing) AB420673 (lane 1), imipenem-resistant (IMP-1-producing) AB421793 (lane 2), and imipenem-sensitive AB420039 (lane 3). Lanes M1 (HindIII/EcoRI-digested phage λ) and M2 (100 bp stepwise ladder) show band patterns of DNA marker fragments (sizes in bp are indicated on the edge of the gel). ERIC-PCR was performed with primers ERIC2 and ERIC1R.

carbapenemase was involved in the imipenem resistance as previously described [16].

ERIC-PCR Analysis

ERIC is based on the analysis of the repetitive chromosomal sequences and has recently been used for clonal characterization of intra- and inter-species of Enterobacteriaceae. Dispersed repetitive DNA sequences in the genomes of bacteria, using conserved primers corresponding to ERIC sequences, were also tested by PCR. ERIC-PCR generates a characteristic genomic fingerprinting that could be used to reveal intra- and inter-species genotypic variations among Enterobacteriaceae [8], *P. aeruginosa* [22] and *A. baumannii* [22, 24, 30]. Two clinical isolates generated 7 to 12 distinct bands by ERIC-PCR, of sizes ranging from 0.1 to 35.5 kb (Fig. 2). The ERIC-PCR fingerprints of two imipenem-resistant (IMP-1-producing) *A. baumannii* isolates were identical and different from those of one imipenem-sensitive isolate. These results were confirmed by PFGE analysis with SmaI (data not shown).

DISCUSSION

Since 2003, a high incidence of imipenem resistance has been observed among nosocomial *A. baumannii* isolates in Korea. Therefore, it was our purpose to ascertain whether the resistance was due to the dissemination of an imipenem resistance determinant and/or to the spread of one *A. baumannii* clone. Microbiological assays showed that imipenem was inactivated by two imipenem-resistant *A. baumannii* isolates. An alteration of their carbapenemase activity was observed in the presence of EDTA, suggesting the production of a metallo-β-lactamase. Two isolates produced a β-lactamase with an apparent pI of 9.0. Based on the IEF result and previous report on IMP-1-producing *A. baumannii* in Italy [26], the IMP-1 metallo-β-lactamase appears to be involved in the imipenem resistance. Because IMP-1-producing *A. baumannii* confers resistance to most β-lactams such as penicillins, extended-spectrum cephalosporins, and carbapenems, a limited number of antimicrobial agents maintain reliable activity against IMP-1-producing *A. baumannii*, including monobactams and sulbactam. Thus, it is important to monitor and control the spread of IMP-1-producing *A. baumannii* conferring resistance to most β-lactams.

We detected two different isolates of imipenem-resistant *A. baumannii* from Korean patients. Two isolates produced IMP-1. The clonal relatedness of *A. baumannii* Korean isolates was evaluated by ERIC-PCR. Both IMP-1-producing isolates presented a very similar antibiotic resistance profile and DNA fingerprinting pattern. Such close similarities of phenotypic and genotypic characteristics can be explained

by a common clonal origin. Our results indicate that the observed imipenem resistance among two Korean *A. baumannii* isolates is due to the spread of an IMP-1-producing clone. Our microbiological test of carbapenemase activity is a simple method for screening of clinical isolates, which produce class B metallo-β-lactamase, to determine their clinical impact and to prevent further spread. The clinical significance of these isolates, which are emerging in Korea, is of great importance, since clinicians are advised against the use of extended-spectrum cephalosporins, imipenem, and aminoglycosides. This observation emphasizes the importance of having effective control measures in Asian hospitals, such as early detection of colonized patients, isolation procedures, and judicious use of antibiotics.

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