Prevalence of multi-drug resistant *Acinetobacter baumannii* from a healthcare facility of Gangwon province in Korea

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ABBREVIATION

ADC	:	*Acinetobacter* derived cephalosporinase
AFLP	:	amplified fragment length polymorphism
AME	:	aminoglycoside modifying enzyme
ARDRA	:	amplified ribosomal DNA restriction analysis
ATCC	:	american type culture collection
DDST	:	double disk synergy test
EDTA	:	ethylenediaminetetraacetic acid
ESACs	:	extended-spectrum AmpCs
ICUs	:	Intensive care units
MBLs	:	metallo β-lactamases
MDR	:	multi-drug resistant
MDRAB	:	multi-drug resistant *Acinetobacter baumannii*
OXAs	:	oxacillinases
PCR	:	polymerase chain reaction
QRDR	:	quinolone resistance-determining region
REP	:	repetitive extragenic palindromic
REP-PCR	:	repetitive extragenic palindromic polymerase chain reaction
TAE	:	Tris acetate EDTA

ABSTRACT

The multidrug-resistant (MDR) *Acinetobacter baumannii* has emerged as a significant infectious agent in hospitals worldwide. *A. baumannii* was previously considered an opportunistic pathogen of relatively low virulence. However, recent reports from various locations around the world suggest that *A. baumannii* is now frequently isolated and is associated with severe infections and adverse outcomes. The purpose of this study was to determine the genetic basis for MDR and the clonal relationship among *A. baumannii* clinical isolates obtained from a university hospital in Gangwon province of Korea. To estimate the prevalence of antibiotic resistance determinants, PCR assays and sequence analysis were performed for the detection of β-lactamases, 16S rRNA methylase, aminoglycoside-modifying enzymes, and quinolone resistance determining regions (QRDR). Amplified fragment length polymorphism (AFLP) and repetitive sequence-based polymerase chain reaction (REP-PCR) were performed to determine the clonal relatedness of MDR *A.*

baumannii. All of the MDR *A. baumannii* isolates were encoded by both *bla*$_{\text{OXA-23-like}}$ and *bla*$_{\text{OXA-51-like}}$ genes and all isolates with the *bla*$_{\text{OXA-23-like}}$ gene had the upstream element IS*Aba*1 to promote increased gene expression and subsequent resistance to carbapenemase. In all *A. baumanni*, those isolates encoded by *bla*$_{\text{AmpC}}$ had IS*Aba*1 inserted upstream of *bla*$_{\text{AmpC}}$. Eighty-one *A. baumannii* were encoded by the *bla*$_{\text{ADC-7-like}}$ gene. To investigate aminoglycoside resistance, 16S rRNA methylase gene *arm*A was detected in 70 (81%) clinical isolates, and phosphotransferase genes encoding *aac(3)-Ia* and *aac(6')-Ib* were the most prevalent. A combination of 16S rRNA methylase and aminoglycoside-modifying enzyme genes (*arm*A, *aac(3)-Ia*, *aac(6')-Ib*, and *aph(3')-Ia*) were found in 45 (52%) isolates. The sequencing results for the QRDR of *gyr*A and *par*C revealed the presence of Ser (TCA) 83 Leu (TTA) and Ser (TCG) 80 Leu (TTG) substitutions in the respective enzymes for all MDR *A. baumannii* isolates sequenced. Moreover, all of the *A. baumannii* isolates showed very similar or identical band patterns on REP-PCR and

AFLP profiles, suggesting that they had originated from a common ancestor and clonally spread in the university hospital in Gangwon province. The clonal spread of MDR *A. baumannii* is a major factor contributing to the growing problem of antimicrobial resistance. Molecular typing for MDR *A. baumannii* could be helpful in confirming the identification of a common source or cross-contamination. This is an important step in enabling epidemiological tracing of these strains.

Key words : multidrug resistant *Acinetobacter baumannii*, $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-51-like}}$, IS*Aba*I, *arm*A, GyrA, ParC, REP-PCR, AFLP

I. INTRODUCTION

Acinetobacter baumannii has emerged as an important nosocomial pathogen in outbreaks of hospital infections and is ranked second after *Pseudomonas aeruginosa* among nosocomial pathogens of aerobic nonfermentative gram-negative bacilli[1, 2]. *A. baumannii* causes respiratory and urinary tract infections, meningitis, endocarditis, burn infections, and wound sepsis, especially in intensive care units (ICUs)[2, 3].

Infections caused by multi-drug resistant (MDR) bacteria occurred throughout the world. During the last decade efforts to combat MDR microorganisms have mainly focused on gram-positive bacteria and drug companies have developed several novel antimicrobial agents to fight these bacteria. Unfortunately, the growing problem of MDR gram-negative bacteria has not been paralleled with the development of new antimicrobial agents. As a result, there are now a growing number of reports on infections caused by MDR gram-negative microorganisms for which no adequate therapeutic options exist.

This return to the pre-antibiotic era has become a reality in many parts of the world. Although the antimicrobial susceptibility pattern of *A. baumannii* is variable, many isolates exhibit resistance to commonly used antimicrobial agents, such as penicillins, cephalosporins, aminoglycosides, and fluoroquinolones by intrinsic and acquired mechanisms. As a consequence of the nature of MDR microorganism, the treatment of MDR *A. baumannii* infections is complicated by the limited choice of effective antimicrobial agents. Carbapenems, mainly imipenem and meropenem, are the most potent β-lactams against gram-negative bacteria and are most widely used for treatment of extended-spectrum-β-lactamase-producing *A. baumannii* and *P. aeruginosa*. However, intensive use of carbapenems has facilitated the emergence of carbapenem-resistant bacteria[4-6].

Furthermore, *A. baumannii* strains that are resistant to all antimicrobial agents tested, but only susceptible to colistin, referred to as extensive-drug resistant strains, were detected in Taiwan[7], Italy[8], Greece[9], and Korea[10-12]. The emergence of pan-

drug resistant *A. baumannii*, resistant to anti-pseudomonal-penicillins, cephalosporins, carbapenems, monobactam, aminoglycosides, fluoroquinolone, tetracyclines, and polymyxins, has also been reported in some country[13, 14].

Carbapenem resistance in *A. baumannii* is mediated most often by oxacillinases (OXAs) and less frequently by metallo β-lactamases (MBLs)[15]. There are four main OXA subgroups associated with *A. baumannii*: the chromosomally located intrinsic OXA-51-like and the acquired OXA-23-like, OXA-40-like and OXA-58-like. OXAs exhibit such weak hydrolysis of carbapenems that they should not allow the development of resistance; however, they are sometimes associated with insertion elements that can increase expression of the carbapenemase[16-18].

A growing number of β-lactamases conferring resistance to expanded-spectrum cephalosporins have been identified in *Acinetobacter* spp. Resistance to oxyiminocephalosporins (ceftazidime and cefotaxime) is usually related to the

overproduction of the resident ampC-type β-lactamase[19, 20] encoded by the gene *bla*$_{ampC}$. The over expression of that gene has been associated with the insertion sequence IS*Aba1* providing a strong promoter[19, 21].

Recently, the AmpC variants have been named according to a nomenclature specific to *A. baumannii* (e.g., ADC, for *Acinetobacter* derived cephalosporinases)[20]. Most AmpC-type β-lactamases naturally produced by gram-negative bacteria hydrolyze amino- and ureidopenicillins, cephamycins (cefoxitin and cefotetan), and, at a lower level, oxyiminocephalosporins, such as ceftazidime, cefotaxime, and ceftriaxone, and monobactams, such as aztreonam[22]. Zwitterionic cephalosporins (cefepime and cefpirome) together with carbapenems are usually excluded from the spectrum of activity of AmpC β-lactamases[23].

However, natural cephalosporinases possessing broadened substrate activity in the taxa Enterobacteriaceae and *P. aeruginosa* have been reported[24-26]. These extended-spectrum AmpCs (ESACs) confer reduced susceptibility to all

cephalosporins[24-26]. They differ from “regular” cephalosporinases by amino acid substitutions or insertions/deletions in four specific regions that are all located in the vicinity of the active site: the Ω-loop, the H-10 helix, the H-2 helix, and the C-terminal extremity of the protein[27].

The best-described mechanism of resistance to quinolones in *Acinetobacter* spp. is mutations in the genes encoding DNA gyrase A (i.e., *gyr*A) and subunit A of topoisomerase IV (i.e., *par*C). The most important mutations, resulting in changes at codon 83 for *gyr*A and at codon 80 for *par*C, have been mapped to a unique location in each of these genes, the quinolone resistance-determining region (QRDR)[28, 29]. Additional mutations in the QRDR of *gyr*A thought to enhance quinolone resistance include mutations at codons for amino acids Gly81, Ala84, and Glu87[28-34]. Another previously described mutation (at the Val101 codon) does not appear to have an effect on the susceptibility profiles[30]. Ancillary mutations in *par*C, at codons for amino acids Gly78 and Glu84, when combined with

the Ser83 and Ser80 codon mutations, also contribute to high-level quinolone resistance[28, 31, 34]. These mutations interfere with target site binding. Similar to aminoglycosides, many quinolones are also substrates for multidrug efflux pumps[35], including the RND-type pump AdeABC[36, 37] and the MATE pump AdeM[38]. Thus far, plasmid-mediated quinolone resistance, mediated by *qnr* genes, has not been reported for *A. baumannii*[39].

Aminoglycosides continue to play an important role in antimicrobial therapy against both gram-negative and gram-positive pathogens, usually in combination with β-lactam agents [40,41]. They bind specifically to 16S rRNA in the 30S ribosomal subunits and inhibit protein synthesis[42]. Resistance to aminoglycosides is most commonly encountered by aminoglycoside modifying enzymes, including acetyltransferases, nucleotidyltransferases, and phosphotransferases[43,44]. More recently, 16S rRNA methylases (ArmA, RmtA, RmtB, RmtC, and RmtD) have been reported among Enterobacteriaceae, *Pseudomonas* spp., and *Acinetobacter* spp.[45, 46]. Aminoglycoside-

modifying enzymes differ in aminoglycosides modified, whereas 16S rRNA methylases confer a high-level resistance to all formulated aminoglycosides. The distribution of aminoglycoside resistance genes is largely dependent on bacterial species, but the combination of aminoglycoside-modifying enzymes is correlated with the use of aminoglycosides in specific geographic regions [47].

Acinetobacter spp. carried various types of aminoglycoside-modifying enzymes[44], but the distribution of particular aminoglycoside-modifying enzymes based on *Acinetobacter* genomic species was not determined. With the emergence of 16S rRNA methylase ArmA in *Acinetobacter* spp.[10, 45, 48, 49], it should be determined whether the prevalence of ArmA in *Acinetobacter* spp. was due to the spread of specific clones or horizontal gene transfer.

For epidemiological and clinical purposes, a rapid and real-time understanding of genomic fingerprinting profiles of MDRAB (multidrug resistant *A. baumannii*) clinical isolates is necessary in order to develop effective strategies to control their

spread[40]. A variety of methods, including biotyping, antibiotic resistance typing[50], comparing outer membrane protein profiles[51], plasmid profiling[52], ribotyping[53], and analyzing restriction fragment length polymorphisms using pulsed-field gel electrophoresis[54, 55], have been used successfully for the typing of *Acinetobacter* spp. These methods can be complex and time-consuming to perform or can rely on unstable phenotypic characteristics.

A PCR-based fingerprinting system that uses consensus primers for the repetitive extragenic palindromic (REP) sequences found in many bacterial chromosomes has been shown to be applicable to a wide range of bacterial species[56-58] and is known as repetitive extragenic palindromic polymerase chain reaction (REP-PCR). The highly conserved REP sequence is approximately 35 nucleotides long, includes an inverted repeat, and can occur in the genome singly or as multiple adjacent copies [59]. REP-PCR has been recognised as an effective method for bacterial genotyping[60, 61]. The method effectively discriminated

between epidemic and sporadic isolates but required time-consuming genomic DNA isolation, purification, and quantitation[40].

The development and application of amplified fragment length polymorphism (AFLP) as a genomic fingerprinting method has more recently led to significant advances in the study of taxonomy and genetic diversity of bacteria[62, 63]. The AFLP technique is based on the selective PCR amplification of restriction fragments from total digest of genomic DNA. It involves two amplification steps: a low-level or preselective amplification, followed by a more selective amplification, which generates a set of fragments that can be used as the discriminatory marker set for a particular sample[64, 65]. AFLP is a well-established species identification and genomic fingerprinting method that has been used successfully to study the epidemiology of *A. baumannii* [66, 67].

The purpose of this study is to determine the genetic basis and molecular epidemiology of these MDR *A. baumannii*

isolates and to determine the clonal relationship among *A. baumannii* clinical isolates obtained from the Gangwon province of Korea. This study investigated the various molecular determinants of *A. baumannii* including carbapenem, cephalosporin, aminoglycoside, and quinolone resistance.

II. MATERIALS AND METHODS

Clinical isolates

A total of eight-six non-duplicate *A. baumannii* isolates were collected from the university hospital laboratory in the Gangwon province of Korea from July 2007 to July 2010. Over the study period, all MDR *A. baumannii* that were resistant to three or more classes of antimicrobial agents, including carbapenems, were collected. The isolates were identified by conventional identification techniques and Microscan walkaway 96 (Dade Behring, Sacramento, CA, USA).

Species identification

Commercial identification systems based on phenotypic tests are unable to identify most *Acinetobacter* species. Amplified ribosomal DNA restriction analysis (ARDRA) is one of the few genotypic methods that has been validated using a large set of reference strains to identify species of the genus *Acinetobacter* [11].

ARDRA was carried out as previously described, using restriction endonucleases (*Cfo*I, *Alu*I, *Mbo*I, *Msp*I, *Rsa*I)[68].

To prepare DNA templates, brain heart infusion broth (Becton Dickinson, Franklin Lake, NJ, USA) was inoculated with one colony of *A. baumannii*. After 24 hours incubation at 37°C, 1 ㎖ of the culture was centrifuged at 12,000 g for 10 minutes. The pellets were resuspended in 1 ㎖ of saline (0.85 NaCl, Duksan, Ansan, Korea) and then harvested by centrifugation. Bacterial pellet were suspended in 200 ㎕ of sterilized distilled water and boiled for 10 minutes. After being centrifuged at 12,000 g for 10 minutes, the supernatant was collected to use as the template DNA[69].

The supernatant (2 ㎕) was added to 48 ㎕ aliquots of polymerase chain reaction (PCR) mixture containing 0.5 U *Taq* polymerase (Cosmo Genetech Co., Seoul, Korea), 100 μM deoxynucleoside triphosphates (dNTPs), and 0.2 μM primer in a reaction buffer (1.5 mM $MgCl_2$ and 50 mM KCl in 10 mM Tris-

HCl, pH 8.3). After initial denaturation at 95°C for 5 min, the reaction mixture was run through 35 cycles of denaturation at 95°C for 45 seconds, annealing at 50°C for 45 seconds, and extension at 72°C for 1 minute. Finally, a seven minutes extension period at 72°C was carried out. The amplification reactions were performed with a thermal cycler (GeneAmp PCR® System 2700, Applied Biosystems, Foster City, CA, USA).

The oligonucleotide primers were derived from conserved regions in the 16S rRNA. The sequences of the primers were 5'-TGGCTCAGATTGAACGCTGGC GGC-3' (5' end of 16S rRNA gene) and 5'-TACCTTGTTACGAC TTCACCCCA-3' (3' end of 16S rRNA gene). Amplicons with a size of 1,500 bp were used as template DNA for the restriction analysis.

Restriction enzyme digestion was carried out for 2 hours at 37°C in 20 ㎕ volumes of commercially supplied incubation buffer containing 5 U of restriction enzyme *Alu*I (AGCT), *Cfo*I (GCGC), *Mbo*I (GATC), *Msp*I (CCGG), *Rsa*I (GTAC), and 10

㎕ of PCR product[68]. Restriction fragment patterns were separated by agarose gel electrophoresis in Tris-acetate-EDTA (TAE) buffer and visualized after being stained with ethidium bromide (0.5 ㎍/㎖). The agarose gel was photographed with the Molecular Imager® Gel Doc™ XR+ Imaging System (Bio-Rad, Hercules, CA, USA) under UV illumination. Identification of product bands was established by molecular weight marker of the 100 bp DNA ladder (Bioneer Co., Daejeon, Korea).

Antimicrobial susceptibility test

Minimal inhibitory concentrations of clinical antimicrobials for all isolates were determined by the Microscan Walk/Away 96 AI system (Dade Behring, Sacramento, CA, USA) and were interpreted according to CLSI M100-S19 (formerly NCCLS, 2009). Twenty-three antimicrobial agents were tested: penicillin (ampicillin), β-lactam/β-lactamase inhibitor combinations (ampicillin/sulbactam, ticarcillin

/clavulanic acid), cephems (ceftazidime, cefepime, cefotaxime, ceftriaxone, cefazolin, cefuroxime, cefotetan), carbapenem (imipenem, meropenem), monobactams (aztreonam), aminoglycosides (gentamicin, tobramycin, amikacin), tetracyclines (tetracycline), fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin), folate pathway inhibitors (trimethoprim-sulfamethoxazole), and ESBLs (ceftazidime/ clavulanic acid, cefotaxime/clavulanic acid).

Screening of carbapenemase activity

In order to analyze the production of class B and D carbapenemases, imipenem-nonsusceptible *A. baumannii* isolates were first screened by a modified Hodge test[70, 71]. The surface of a Muller-Hinton agar plate was inoculated with an overnight culture suspension of *Escherichia coli* ATCC 25922. An imipenem disk was placed at the center of the plate and imipenem-nonsusceptible *Acinetobacter* was streaked heavily 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 result. An imipenem-EDTA double disc synergy test (DDST) was performed to screen for the production of metallo-β-lactamases (MBL)[71, 72].

An overnight culture of the modified Hodge test-positive strains was inoculated on a Muller-Hinton agar plate. The imipenem disk (30 μg) and a blank filter paper disk were placed 15 mm apart from edge to edge, and 10 $\mu\ell$ of 0.5 M EDTA solution was applied to the blank disk, which resulted in approximately a 1.5 mg/disk. After overnight incubation, the presence of an enlarged zone of inhibition was interpreted as a positive result, which shows the inactivation of class B MBL activity by EDTA[73].

PCR amplification of β-lactamase genes

The β-lactamases and resistance determinants were detected by PCR, including Ambler class A ESBL genes (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{VEB}$, *bla*$_{KPC}$ and *bla*$_{PER}$), Amber class B MBL

genes (*bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{SIM}$), an Amber class C AmpC β-lactamase genes (*bla*$_{ADC}$), and Amber class D carbapenemase genes (*bla*$_{OXA\text{-}23\text{-}like}$, *bla*$_{OXA24\text{-}like}$, *bla*$_{OXA\text{-}51\text{-}like}$, and *bla*$_{OXA\text{-}58\text{-}like}$).

To detect ESBLs and carbapenemase were amplified by multiplex PCR method[74]. Genes coding for ESBLs were sought by PCR using primers specific for the genes *bla*$_{TEM\text{-}like}$, *bla*$_{SHV\text{-}like}$, *bla*$_{OXA\text{-}like}$, *bla*$_{VIM\text{-}like}$, *bla*$_{IMP\text{-}like}$, *bla*$_{KPC\text{-}like}$, *bla*$_{VEB\text{-}like}$, *bla*$_{PER\text{-}like}$, and *bla*$_{GES\text{-}like}$ (Table 1).

PCR amplification of resistant determinants was performed in a 50 ㎕ volume containing: 2 ㎕ of boiled bacterial suspensions, 20 pM of each primer, 200 μM of dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.5 U of *Taq* DNA polymerase (Cosmo Genetech Co., Seoul, Korea). Amplification was carried out as follows: initial denaturation at 94°C for 10 minutes; 30 cycles of 94°C for 40 seconds, 60°C for 40 seconds and 72°C for 1 minute; and a final elongation step at 72°C for 7 minutes. For the carbapenemase gene multiplex PCR

assays, the annealing temperature was optimal at 55°C for amplification of *bla*$_{VIM}$, *bla*$_{IMP,}$ and *bla*$_{KPC}$ genes.

A multiplex polymerase chain reaction (PCR) assay was performed for the detection of the carbapenem resistant genes found in the *A. baumannii* isolates according to the method described[75]. These primers were combined with eight primers that were designed to amplify fragments of genes encoding for *bla*$_{OXA-23-like}$, *bla*$_{OXA-24-like}$, *bla*$_{OXA-58-like}$, and *bla*$_{OXA-51-like}$ carbapenemase (Table 2). The amplification conditions were as follows: initial denaturation at 94°C for 5 minutes, 30 cycles of 94°C for 25 seconds, 52°C for 40 seconds, 72°C for 50 seconds, and a final elongation at 72°C for 6 minutes.

The presence of IS*Aba*1 inserted upstream of *bla*$_{OXA-23-like}$ and *bla*$_{OXA-51-like}$ like was sought via PCR, using combinations of the IS*Aba*1 primers and the OXA-23-like and OXA-51-like reverse primers. An annealing temperature of 58°C was used for the ISAba1F/OXA-23-likeR and IS*Aba*1/OXA-51-likeR PCRs[17].

For detection of extended-spectrum cephalosporinase, primers PreAmpC-PISAba1 and PreAmpC-Ab1 were used in combination with primer PreAmpC-Ab2 to amplify 1,521 bp and 1,254 bp fragments, respectively, encompassing the entire *bla*$_{\text{AmpC}}$ gene with and without the PIS*Aba*1 promoter, respectively (Table 2)[27]. For detection of the *bla*$_{\text{ADC-7-like}}$ gene, the primer pair of EBF blaADC-7 and EBR blaADC-7 was used[32] (Table 3). Amplification was carried out as follows: initial denaturation at 94°C for 10 minutes; 30 cycles of 94°C for 30 seconds, 50°C for 1 minute and 72°C for 1 minute; and a final elongation step at 72°C for 10 minutes.

Table 1. Oligonucleotide sequence for detection of β-lactam resistance genes

Target gene	Primer	Oligonucleotide sequence (5' to 3')	Reference
bla$_{\text{TEM-like}}$	MultiTSO-T-F	CATTTCCGTGTCGCCCTTATTC	Dallenne *et al*.
	MultiTSO-T-R	CGTTCATCCATAGTTGCCTGAC	
bla$_{\text{SHV-like}}$	MultiTSO-T-F	AGCCGCTTGAGCAAATTAAAC	
	MultiTSO-T-R	ATCCCGCAGATAAATCACCAC	
bla$_{\text{OXA-like}}$	MultiTSO-O-F	GGCACCAGATTCAACTTTCAAG	
	MultiTSO-O-R	GACCCCAAGTTTCCTGTAAGTG	
bla$_{\text{VEB-like}}$	MultiVEB-F	CATTTCCCGATGCAAAGCGT	
	MultiVEB-R	CGAAGTTTCTTTGGACTCTG	
bla$_{\text{PER-like}}$	MultiPER-F	GCTCCGATAATGAAAGCGT	
	MultiPER-R	TTCGGCTTGACTCGGCTGA	
bla$_{\text{GES-like}}$	MultiGES-F	AGTCGGCTAGACCGGAAAG	
	MultiGES-R	TTTGTCCGTGCTCAGGAT	
bla$_{\text{IMP-like}}$	MultiIMP-F	TTGACACTCCATTTACDG[a]	
	MultiIMP-R	GATYGAGAATTAAGCCACYCT[a]	
bla$_{\text{VIM-like}}$	MultiVIM-F	GATGGTGTTTGGTCGCATA	
	MultiVIM-R	CGAATGCGCAGCACCAG	
bla$_{\text{KPC-like}}$	MultiKPC-F	CATTCAAGGGCTTTCTTGCTGC	
	MultiKPC-R	ACGACGGCATAGTCATTTGC	
bla$_{\text{PER-1}}$	PER1-925F	ATGAATGTCATTATAAAAGC	This study
	PER1-925R	AATTTGGGCTTAGGGCAGAA	

[a]Y=T or C; R=A or G; S=G or C; D=A or G or T.

Table 2. Oligonucleotide sequence for detection of OXA type carbapenemase gene

Target gene	Primer	Oligonucleotide sequence (5' to 3')	Reference
bla$_{\text{OXA-23-like}}$	OXA23-F	GAT CGG ATT GGA GAA CCA GA	Woodford *et al.*
	OXA23-R	ATT TCT GAC CGC ATT TCC AT	
bla$_{\text{OXA-24-like}}$	OXA24-F	GGT TAG TTG GCC CCC TTA AA	
	OXA24-R	AGT TGA GCG AAA AGG GGA TT	
bla$_{\text{OXA-51-like}}$	OXA51-F	TAATGCTTTGATCGGCCTTG	
	OXA51-R	TGG ATT GCA CTT CAT CTT GG	
bla$_{\text{OXA-58-like}}$	OXA58-F	AAG TAT TGG GGC TTG TGC TG	
	OXA58-R	CCC CTC TGC GCT CTA CAT AC	
IS*Aba*1	ISAba1F	CAC GAA TGC AGA AGT TG	Segal *et al.*
	ISAba1R	CGA CGA ATA CTA TGA CAC	

Table 3. Oligonucleotide sequence for detection of cephalosporinase gene

Target	Primer	Oligonucleotide sequence (5' to 3')	Reference
*bla*ADC	PreAmpC-PISAba1	GACCTGCAAAGAAGCGCTGC	Rodríguez-Martínez *et al.*
	PreAmpC-Ab1	GAGCTAATCATGCGATTTAAA	
	PreAmpC-Ab2	GCTTAGGATATGTTTGGTTCTT	
*bla*ADC-7	EBF *bla*ADC-7	GGGATATCATGCGATTTAAAAAAATTTC	Hujer *et al.*
	EBR *bla*ADC-7	AAGGATCCTTATTTCTTTATTGCATTC	

Detection of aminoglycoside resistance genes

To detect 16S rRNA methylase genes, specific primers for *arm*A, *rmt*A, *rmt*B, *rmt*C, *rmt*D and *npm*A were used. The PCR primers used in this study are listed in Table 4.[41]. The reaction was carried out in a total volume of 20 $\mu\ell$, containing 2 $\mu\ell$ of template and PCR buffer (40 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$), 200 μM of dNTPs, 20 pM of each primer, and 0.5 U of G-*Taq* DNA polymerase. Amplification conditions were as follows: 94°C for 5 minutes, 25 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 1 minute;, and a final extension at 72°C for 10 minutes.

To detect the genes encoding the following aminoglycoside-modifying enzymes were investigated: acetyltransferases AAC(3)-Ia, AAC(3)-IIa, AAC(6')-Ib, and AAC(6')-1h;, nucleotidyltransferases ANT(2")-Ia;, and phosphotransferases APH(3')-Ia and APH(3')-VI. Three different multiplex PCRs were conducted. Triplex assay 1 included primers for the amplification of *aac(3')-Ia*, *aac(3')-IIa*, and

aac(6')-Ih. Duplex assay 2 included primers for the amplification of *aph(3')-VI* and *ant(2')-Ia*. Duplex assay 3 included primers for the amplification of *aph(3')-Ia* and *aac(6')-Ib*[76].

A PCR was performed in a 20 ㎕ volume containing: 2 ㎕ of boiled bacterial suspensions, 10 pM of each primer, 200 μM of dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.5 U of *Taq* DNA polymerase (Cosmo Genetech Co., Seoul, Korea). Amplification was carried out as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 95°C for 30 seconds, 49°C (triplex 1 and duplex2) or 54°C (duplex 3) for 1 minute and 72°C for 1 minute; and a final elongation step at 72°C for 5 minutes.

Table 4. Oligonucleotide sequence for aminoglycoside resistance genes and 16S rRNA methylase genes

Target	Primer	Oligonucleotide sequence (5' to 3')	Reference
*arm*A	armA-F	TATGGGGGTCTTACTATTCTGCCTAT	Fritsche *et al*
	armA-R	TCTTCCATTCCCTTCTCCTTT	
*rmt*A	rmtA-F	CTAGCGTCCATCCTTTCCTC	
	rmtA-R	TTTGCTTCCATGCCCTTGCC	
*rmt*B	rmtB-F	TCAACGATGCCCTCACCTC	
	rmtB-R	GCAGGGCAAAGGTAAAATCC	
*rmt*C	rmtC-F	GCCAAAGTACTCACAAGTGG	
	rmtC-R	CTCAGATCTGACCCAACAAG	
*rmt*D	rmtD-F	CTGTTTGAAGCCAGCGGAACGC	
	rmtD-R	GCGCCTCCATCCATTCGGAATAG	
*npm*A	npmA-F	CTCAAAGGAACAAAGACGG	
	npmA-R	GAAACATGGCCAGAAACTC	
aac(3)-Ia	1a-F	GACATAAGCCTGTTCGGTT	Akers *et al*
	1a-R	CCCGCTTTCTCGTAGCA	
aac(3)-IIa	1b-F	ATGCATACGCGGAAGGC	
	1b-R	TGCTGGCACGATCGGAG	
aac(6)-Ih	1c-F	TGCCGATATCTGAATC	
	1c-R	ACACCACACGTTCAG	
aph(3)-VI	2a-F	CGGAAACAGCGTTTTAGA	
	2a-R	TTCCTTTTGTCAGGTC	
ant(2)-Ia	2b-F	ATCTGCCGCTCTGGAT	
	2b-R	CGAGCCTGTAGGACT	
aph(3)-Ia	3a-F	CGAGCATCAAATGAAACTGC	
	3a-R	GCGTTGCCAATGATGTTACAG	
aac(6)-Ib	3b-F	TATGAGTGGCTAAATCGAT	
	3b-R	CCCGCTTTCTCGTAGCA	

Determination of quinolone resistance by PCR amplification and DNA sequencing

Amplification of the *gyr*A and *par*C genes for direct sequencing was performed using the forward and reverse primers listed in Table 5. PCR conditions included an initial denaturation at 95°C for 1 minute followed by 30 cycles of 95°C for 30 seconds and annealing for 45 seconds at 54°C for both *gyr*A and *par*C, with an extension at 72°C for 30 seconds. Cycling was followed by a final extension at 72°C for 5 minutes.

Sequencing of the PCR products was carried out at Cosmo Genetech (Seoul, Korea). Sequencing reactions were performed using nested primers (Table 6) and the resulting consensus sequences were compared to GenBank (www.ncbi.nlm.nih.gov/GenBank) reference sequences X82165 for *gyr*A and X95819 for *par*C of *A. baumannii* [32].

PCR amplifications with the primers for efflux genes (*ade*B, *ade*J, and *abe*M) were performed[40]. Amplification conditions were as follows: 94°C for 5 minutes; 25 cycles of

94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 45 seconds; and a final extension at 72°C for 7 minutes.

Table 5. Oligonucleotide sequence used to amplify the *gyr*A and *par*C genes for sequence analysis

Target gene	Primer	Oligonucleotide sequence (5' to 3')	Reference
*gyr*A	gyrA-F	ATGAGCGTATCGGAAATCC 6	Hujer *et al*.
	gyrA-R	ATCAATCCTTCAATCGAGATATTC	
	gyrA-SF	GGAAATCCGACCGATTGCC	
	gyrA-SR	CGAGATATTCGGATTGTCAGC	
*par*C	parC-F	ATGGAAGATAAGCTGACTATG	
	parC-R	GTTGGTAAATCCGGAGC	
	parC-SF	GAAGATAAGCTGACTATGACCAG	
	parC-SR	GAGCCGGAATATATTCAGC	

Table 6. Oligonucleotide sequence used to amplify the *adeB*, *ade*J and *abeM* genes of efflux pump

Target gene	Primer	Oligonucleotide sequence (5' to 3')	Reference
*ade*B	adeB-F	GTATGAATTGATGCTGC	Magnet *et al*.
	adeB-R	CACTCGTAGCCAATACC	
*ade*J	adeJ-F	TTCTTTGGTGGTACAACAGG	Zhong-Qiang *et al*.
	adeJ-R	GCTGCAATCAGTTTCTCATG	
*abe*M	abeM-F	TGCAACGCAGTTTCATTTTT	Bratu *et al*.
	abeM-R	CGATGTTTCATCGGCTTTTT	

REP- PCR

To prepare DNA templates, brain heart infusion broth (Becton Dickinson, Franklin Lake, NJ, USA) was inoculated with one colony of *A. baumannii*. After 24 hours incubation at 37°C, 1 ㎖ of the culture was centrifuged at 12,000 g for 10 minutes. The pellets were resuspended in 1 ㎖ of saline (0.85% NaCl) and then harvested by centrifugation. Bacterial genomic DNA was extracted using the DOKDO-Prep® Genomic DNA Purification Kit (ELPIS-Biotech., Inc., Daejeon, Korea), and kept at -20°C until use.

With the REP-PCR method, the primer pair REP1 (5'-IIIGCGCCGICATCAGGC-3') and REP2 (5'-ACGTCTTATC AGGCCTAC-3') was used to amplify putative REP-like elements in the genomic bacterial DNA. The primer REP1 has the nucleotide inosine at ambiguous positions in the REP consensus sequences. Inosine contains the purine base hypoxanthine and is

able to base pair with A, C, G, and T [77]. The 20 ㎕ of PCR mixture contained 2 ㎕ template DNA, PCR buffer (40 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$), 2.5 mM(each) dNTP, and 0.5 U of SP-*Taq* DNA polymerase (Cosmo Genetech Co., Seoul, Korea). The Mg^{2+} concentration was 3 mM and primers were used at 20 μM concentration[77]. The amplification was performed in a thermal cycler (GeneAmp® PCR System 2700, Perkin-Elmer Cetus, Boston, USA) beginning with an initial denaturation step at 94°C for 10 minutes; followed by 30 cycles of 94°C for 1 minute, 45°C for 1 minute, and 72°C for 2 minutes; and ending with a final extension step at 72°C for 16 minutes followed by a hold at 4°C. The PCR products were analyzed using 2.5% agarose gel electrophoresis with 0.5 ㎍/㎖ of ethidium bromide and visualized under UV light.

Fingerprinting by AFLP

AFLP analysis was performed using the established procedures with minor modifications[62, 78]. Briefly, the genomic DNA (1 μg) was digested with 10 U *Hind*III (Fermentas, Burlington, Canada) and *Apa*I (Fermentas, Burlington, Canada). For the ligation of the adapters, 5 pmol of *Hind*III adapter (ADH), 5 pmol of *Apa*I adapter (Apa) and 10 Weiss unit of T4 DNA ligase (Fermentas, Burlington, Canada) were added. The primer used in the PCR step included the respective restriction enzyme and the adapter sequence plus one extension base for primer (A for *Hind*III primer). The DNA bands were analyzed using the Quantity One version 4.5.0 program (Bio-Rad, Hercules, CA, USA) and the similarity index by dendrogram for genetical similarity to the band pattern of each clinical isolates was examined.

III. RESULTS

Clinical isolates

Eight-six strains of non-duplicated *A. baumannii* were identified by MicroScan Walkaway 96 as belonging to *Acinetobacter baumannii*/*haemolyticus*. Genomic identification was performed using the ARDRA method. The experimental results revealed that all strains tested were *A. baumannii*. They were isolated from abscess (n=2), culture of blood (n=3), bronchial washing (n=9), catheter (n=2), cerebrospinal fluid (n=2), drain (n=2), endotracheal aspiration (n=1), sputum (n=47), tip (n=1), urine (n=6), and wound (n=10).

Antimicrobial Susceptibilities

All the isolates showed resistance to ampicillin, cefazolin, cefotaxime, cefotetan, ceftazidime, ceftriaxone, cefuroxime, ciprofloxacin, meropenem, moxifloxacin and ticarcillin/K clavulanate, Moreover, all the isolates showed 99% resistance to cefepime, 98% resistance to gentamicin, 93%

resistance to trimethoprim/sulfamethoxazole, 91% resistance to tobramycin, 88% resistance to aztreonam, 86% resistance to amikacin, 85% resistance to imipenem, and 73% resistance to ampicillin /sulbactam,.

Antimicrobial resistance mechanisms

All of the eighty-six *A. baumannii* isolates showed positive results in the modified Hodge test and negative results in the EDTA disk synergy test, indicating the production of class D or another type of carbapenemase.

All the isolates possessed the encoding gene for an intrinsic $bla_{\text{OXA-51-like}}$ carbapenemase and an acquired $bla_{\text{OXA-23-like}}$ carbapenemase (Figure 1). IS*Aba1* inserted in the upstream of $bla_{\text{OXA-23-like}}$ was identified in all the *A. baumannii* isolates. Among the $bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-51-like}}$ carbapenemase producing *A. baumannii* isolates, thirty-one isolates carried $bla_{\text{TEM-like}}$ and thirty-two isolates carried $bla_{\text{PER-1}}$, respectively (Table 7).

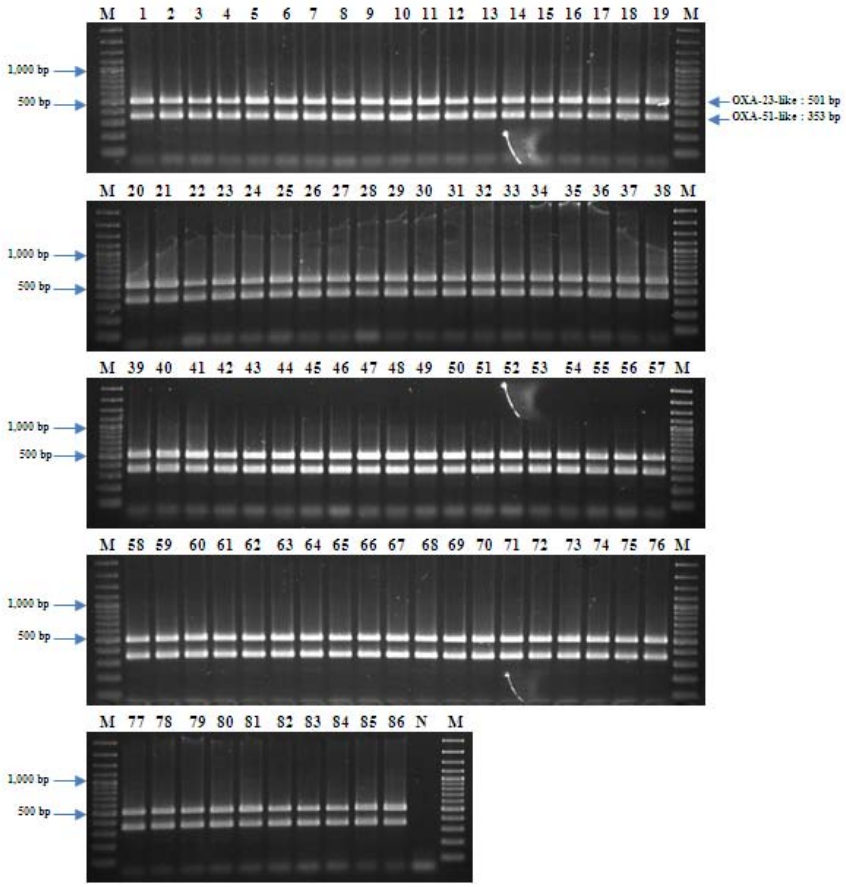

Figure 1. Detection of gene encoding OXA type cabapenemase by multiplex PCR. Lane M, 100 bp Plus DNA ladder (Fermentas, Burlington, Canada).

Table 7. Distribution of β-lactamase gene in *A. baumannii* isolates

Genotype	Number (%) of isolates (n=86)
$bla_{\text{TEM-like}}$	31 (36.0)
$bla_{\text{PER-1}}$	32 (37.2)
$bla_{\text{OXA-23-like}}$	86 (100.0)
$bla_{\text{OXA-51-like}}$	86 (100.0)
$bla_{\text{OXA-23-like}}$ + $bla_{\text{OXA-51-like}}$	86 (100.0)
IS*Aba*1 + $bla_{\text{OXA-23-like}}$	86 (100.0)
$bla_{\text{PER-1}}$ + IS*Aba*1 + $bla_{\text{OXA-23-like}}$	32 (37.2)
$bla_{\text{ADC-like}}$	86 (100.0)
$bla_{\text{ADC-7-like}}$	81 (94.2)
IS*Aba*1 + $bla_{\text{ADC-like}}$	86 (100.0)

Detection of extended-spectrum cephalosporinase

Using the PCR method with the primer pair of PreAmpC-PISAba1 and PreAmpC-Ab2, all the *A. baumannii* isolates were found to be positive for the IS*Aba*1. IS*Aba*1 inserted in the upstream of bla_{AmpC} identified in all *A. baumannii* isolates. PCR amplification nucleotide sequencing analysis using the primer pair of PreAmpC-Ab1 and PreAmpC-Ab2 showed 1,254 bp of DNA fragment as expected.

The experimental results of $bla_{\text{ADC-7-like}}$ gene amplification suggested that eighty-one *A. baumannii* isolates had the $bla_{\text{ADC-7-like}}$ gene (Table 7).

Distribution of aminoglycoside resistance genes in *A. baumannii* isolates

PCR was performed to examine the genetic basis of resistance to aminoglycosides. Three different genes encoding aminoglycoside-modifying enzymes were identified : *aac(3)-Ia* and *aac(6')-Ib* were the most prevalent, found in sixty-eight

isolates, followed by *aph(3')-Ia* in forty-nine isolates. The combination of *aac(3)-Ia*/*aac(6')-Ib*/*aph(3')-Ia* was also founded in forty-nine (57%) isolates and *aac(3)-Ia*/*aac(6')-Ib* in seventeen isolates.

The 16S rRNA methylase gene *arm*A was detected in seventy clinical isolates, but *rmt*A, *rmt*B, *rmt*C, *rmt*D, and *npm*A were not detected in any *A. baumannii* isolate tested. A majority of aminoglycoside resistance genes in *A. baumannii* was the combination of *arm*A/*aac(3)-Ia*/*aac(6')-Ib*/*aph(3')-Ia* indentified in forty-five isolates (Table 8) .

Table 8. Distribution of aminoglycoside resistance genes in *A. baumanii*

Genotype	Number (%) of isolates (n=86)
armA	70 (81.4)
aac(3)-Ia	68 (79.0)
aac(3)-IIa	0 (0.0)
aac(6')-Ih	0 (0.0)
aph(3')-VI	0 (0.0)
ant(2'')-Ia	0 (0.0)
aph(3')-Ia	49 (56.9)
aac(6')-Ib	68 (79.0)
aac(3)-Ia / aac(6')-Ib	17 (19.7)
aac(3)-Ia / aac(6')-Ib/aph(3')-Ia	49 (56.9)
armA/aac(3)-Ia/aac(6')-Ib/aph(3')-Ia	45 (52.3)
None present	12 (13.9)

Determination of quinolone resistance in *A. baumannii*

The sequencing results for the QRDR of *gyr*A and *par*C, encoding DNA gyrase and DNA topoisomerase IV, respectively, revealed substitutions of serine83 to leucine and serine80 to leucine in the respective enzymes in all quinolone-resistant isolates sequenced. All eight-six isolates that harbored amino acid substitution in *gyr*A and *par*C genes exhibited quinolone resistance. No additional amino acid changes were observed for the GyrA polypeptide in all of the isolates. However, nucleotide sequence change were observed in the codon of GyrA at 71^{th} (GGT to GGG) and 162^{th} (CGA to CGT) in all the isolates and in the codon of ParC at 109^{th} (CCT to CCA), 124^{th} (AAA to AAG) and 126^{th} (TCG to TCA) in twenty-six *A. baumannii* isolates (Table 9).

In addition, this analysis shows a high distribution of the efflux pump gene *ade*B and *ade*J in *A. baumannii* isolates. *Ade*J

gene was identified in all the isolates but *ade*B gene in eighty-five isolates.

Table 9. Distribution of quinolone resistant genotype in *A. baumanii*

Target gene	Genotype	Mutation	Number (%) of isolates
	Ser 71	GGT → GGG	86 (100.0)
*gyr*A	Ser 83 to Leu	T**C**A → T**T**A	86 (100.0)
	Arg 162	CGA → CGT	86 (100.0)
	Ser 80 to Leu	T**C**G → T**T**G	86 (100.0)
	Pro 109	CCT → CCA	60 (69.8)
*par*C	Pro 109	CCT → CCG	26 (30.2)
	Lys 124	AAA → AAG	26 (30.2)
	Ser 126	TCG → TCA	26 (30.2)

Clonal relationship of multi-drug resistant *A. baumannii*

AFLP and REP-PCR were performed to determine the clonal relatedness of carbapenemase-producing *A. baumannii*. Agarose gel electrophoresis of the DNA fragments amplified by AFLP and REP-PCR of representative *A. baumannii* isolates is shown in Figure 2 and 4. Most of the MDR *A. baumannii* isolates showed an identical band pattern. The profile generated with AFLP and REP-PCR primers contained several bands, ranging in size from 0.1 to 3 kb. *A baumannii* ATCC 19606 strain tested showed a different band pattern from those of MDR *A. baumannii* clinical isolates.

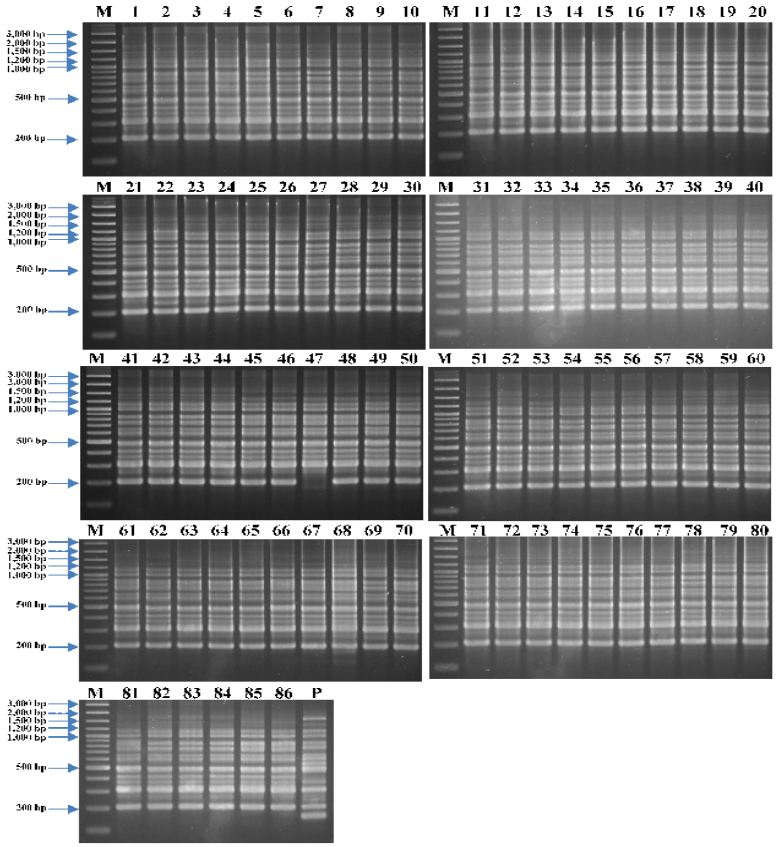

Figure 2. AFLP fingerprints of MDR *A. baumannii*.

Lane M, 100 bp Plus DNA Ladder (Fermentas, Burlington, Canada); lane P, *A. baumannii* ATCC 19606.

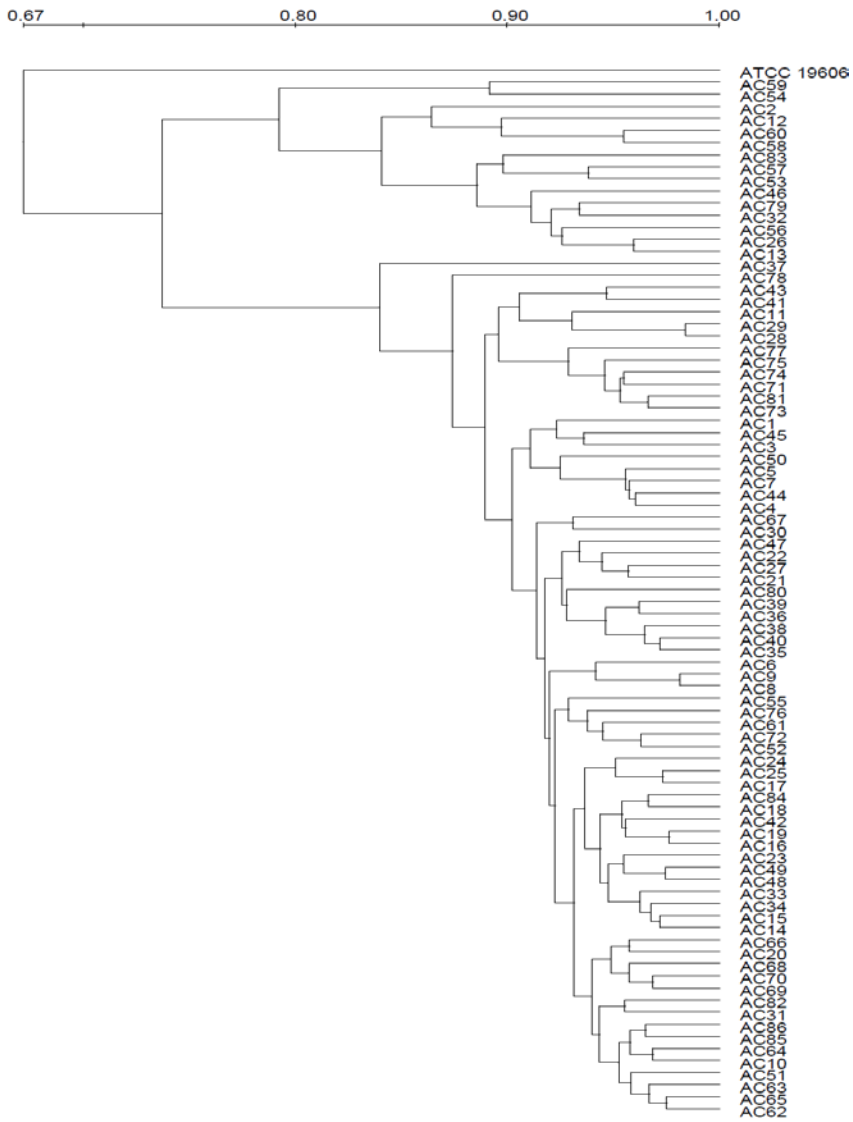

Figure 3. Dendrogram of the similarity index among eighty-six *A. baumannii* isolates provided by AFLP analysis.

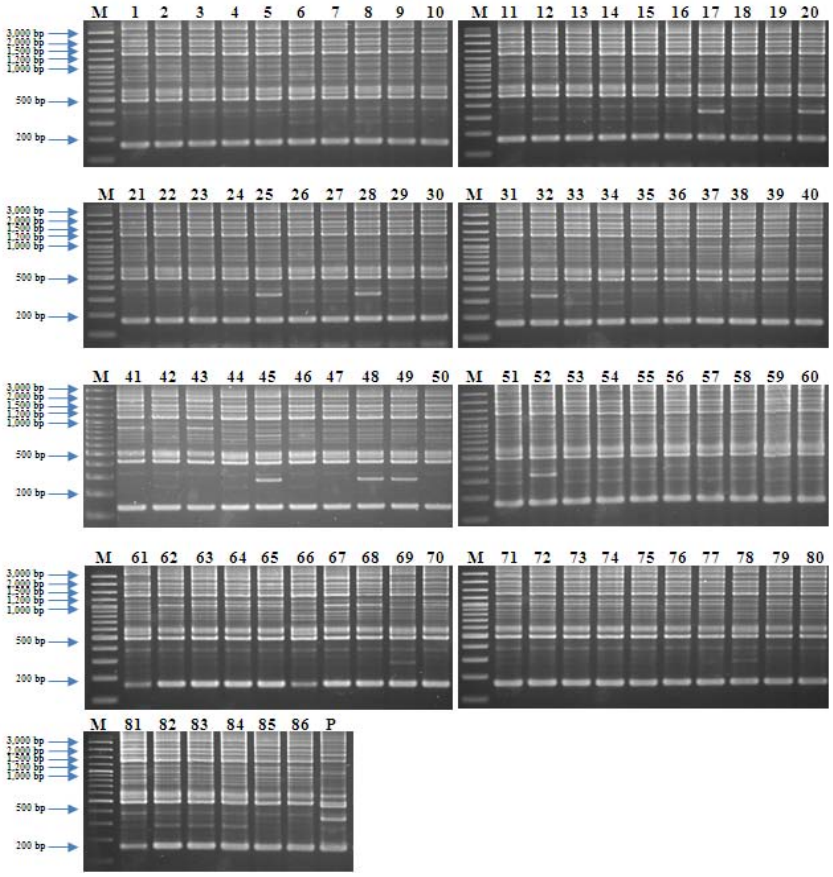

Figure 4. REP-PCR for MDR *A. baumannii*.

Lane M, 100 bp Plus DNA Ladder (Fermentas, Burlington, Canada); lane P, *A. baumannii* ATCC 19606.

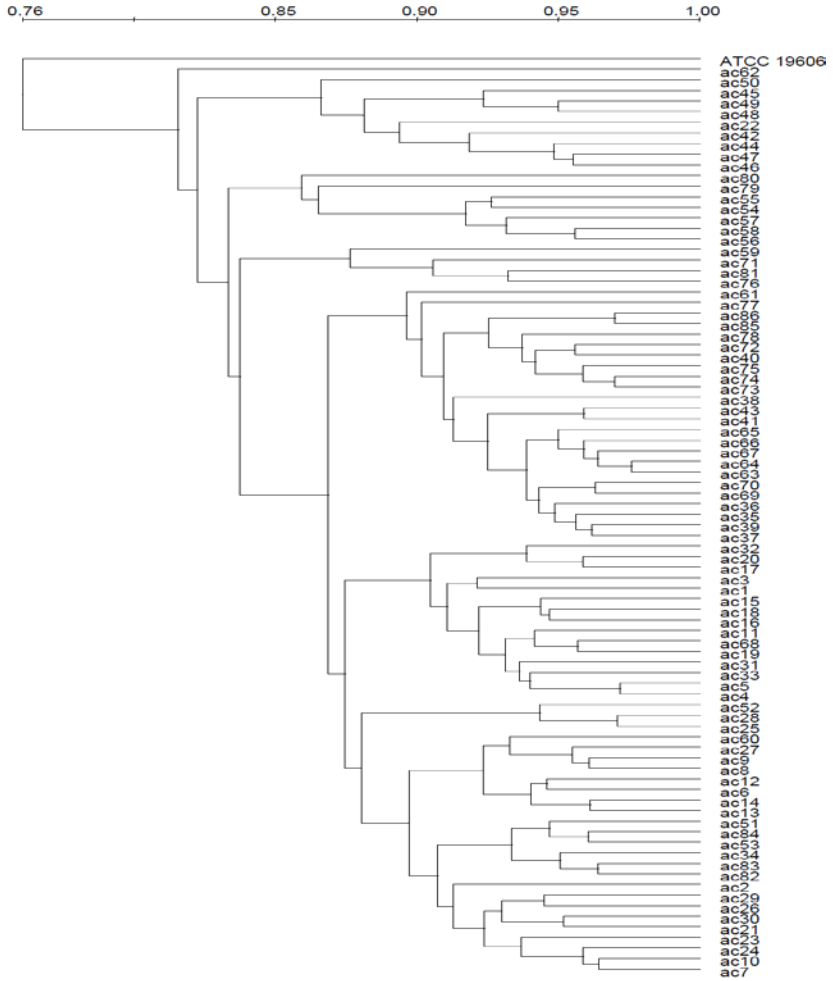

Figure 5. Dendrogram of the similarity index among eighty-six *A. baumannii* isolates provided by REP-PCR analysis.

IV. DISCUSSION

A. baumannii is an increasingly important nosocomial pathogen, frequently causing nosocomial outbreaks in intensive care units of hospital. Most outbreak strains are highly resistant to a variety of antibiotics, and therefore therapeutic options are becoming increasingly limited[17]. Hence, protocols and systems for MDR resistance surveillance has become increasingly important[79].

The aim of this study was to examine the prevalent types of MDR *A. baumannii* and related trends of antimicrobial resistance in a university hospital in the Gangwon province of Korea. For this study, *A. baumannii* clinical isolates were collected from July 2007 to July 2010 and then identified to the species level by ARDRA. Furthermore, the molecular determinants of resistance to carbapenem, cephalosporin, aminoglycoside, and quinolone were investigated. AFLP analysis

and REP-PCR were performed to verify strain relatedness as an indication of prevalence of strains, too.

Nationwide Korean surveillance in 2004 showed that *Acinetobacter* spp. was the seventh most common pathogen, and especially resistance to imipenem had risen from 5% (2001) to 17%[80] (2004). This increase was more dramatic than the case of *Pseudomonas aeruginosa*, where imipenem resistance rose from 19% to 24%. MBLs have received serious attention as a source of carbapenem resistance in Korea, but an outbreak in 2003 involving thirty six patients was caused by *A. baumannii* with OXA-23-like enzyme. This was the first report in Asia[2]. Most isolates with OXA-23-like were from the Southeast region of Korea, while those with OXA-51-like were relatively more prevalent in the Northern region[81].

An outbreak of intrinsic OXA-51-like carbapenemase and acquired OXA-23-like carbapenemase-producing *A. baumannii* was reported in a university hospital of Busan city from November 2006 to July 2007. All isolates analyzed had an

identical band pattern, so they appeared to be caused by the spread of a clonally related epidemic[82]. In addition, an investigation of the genetic determinants of MDR *A. baumanni*i from three university hospitals in the Chungcheong province reported that MDR *A. baumannii* were containing the $bla_{\text{OXA-23-like}}$ and the $bla_{\text{OXA-51-like}}$ carbapenemase genes [83].

In this study, eight-six isolates of imipenem-nonsusceptible *A. baumannii* was confirmed to produce carbapenemase by the modified cloverleaf synergy test. Meanwhile, metallo-β-lactamase-producing isolates (EDTA disk synergy test positive) were not detected. The results suggest that all the *A. baumannii* isolates do not produce class B metallo-β-lactamases, but produce other type of β-lactamases. All of the eighty-six *A. baumannii* isolates carried both the $bla_{\text{OXA-23-like}}$ and the $bla_{\text{OXA-51-like}}$ genes, and all isolates with the $bla_{\text{OXA-23-like}}$ gene had IS*Aba*1 upstream of the $bla_{\text{OXA-23-like}}$ gene (Table 7). This result indicates that acquired OXA-23-like and an intrinsic OXA-51-like carbapenemase-producing *A. baumannii* were prevalent in

the university hospital of Gangwon province during the research period.

Moreover, all the *A. baumannii* strains carried $bla_{\text{OXA-23-like}}$ and $bla_{\text{OXA-51-like}}$ for resistance to carbapenems, whereas thirty-four isolates carried $bla_{\text{PER-1}}$ for resistance to extended-spectrum β-lactams and *arm*A for resistance to high-level of aminoglycosides. Because $bla_{\text{OXA-23-like}}$-producing *A. baumannii* confers resistance to most β-lactams such as imipenem, aztreonam, ceftazidime, and cefepime, a limited number of antimicrobial agents maintain reliable activity against $bla_{\text{OXA-23-like}}$ -producing *A. baumannii*, including polymyxin, sulbactam, and minocycline[84]. Thus, it is important to monitor and control the spread of $bla_{\text{OXA-23-like}}$-producing *A. baumannii* conferring resistance to most β-lactams.

One of the most important mechanisms of aminoglycoside resistance is post-transcriptional rRNA methylation by 16S rRNA methylases. In this study, 16S rRNA methylase gene *arm*A was detected in seventy (81%) clinical

isolates, but *rmt*A, *rmt*B, and *rmt*C were not detected in any *A. baumannii* isolates. According to the multiplex PCR analysis, seven different types of aminoglycoside-modifying enzymes (AME)-encoding genes were present singly or in combination (Table 4). Forty-nine isolates (57%) had one AME gene encoding *aph(3')-Ia* (phosphotransferase), sisty-eight isolates (79%) had two AME genes encoding *aac(3)-Ia* and *aac(6')-Ib* (acetyltransferase), forty-nine isolates (57%) had three AME genes encoding *aac(3)-Ia*, *aac(6')-Ib,* and *aph(3')-Ia,* and seventeen isolates (20%) had no AME gene. Genes encoding *aac(3)-Ia* and *aac(6')-Ib* were the most prevalent. In addition, a combination of 16S rRNA methylase and AME genes (*arm*A, *aac(3)-Ia*, *aac(6')-Ib*, and *aph(3')-Ia*) was found in 45 (52%) isolates (Table 8). All of the seventeen non-presenting isolates were resistant to gentamicin. However, seven isolates were sensitive and one isolate was intermediately resistant to tobramycin. Three were isolates sensitive and seven isolates were intermediately resistant to amikacin. Previous studies in Korea

have reported a different prevalence of AME genes in a polyclonal group of *A. baumannii* isolates : *aac(3)-Ia* (15%), *aac(6')-Ib* (84%), *ant(3")-Ia* (85%), *aph(3')-Ia* (89%), and *aph(3')-VI* (2%) [42]. Contrastively, nucleotidyltransferase [ant(2")-Ia and ant(3"-Ia)] was not detected in any isolates in the present study. This result showed that *A. baumannii* containing nucleotidyltransferase was not prevalent in Gangwon province in Korea.

A major mechanism of quinolone resistance in gram-negative bacteria involves structural changes of the drug targets of DNA gyrase and DNA topoisomerase IV[85]. In *A. baumannii*, the most frequent amino acid substitutions occur at position 83 (Ser-83) of GyrA and at position 80 (Ser-80) of ParC[33]. Sequencing results of previous studies have revealed concurrent mutations in *gyr*A (Ser-83) and *par*C (Ser-80 or Glu-84) in all isolates collected from Chungcheong Province of Korea[83]. In this study, all of the isolates that harbored amino acid substitution in GyrA and ParC polypeptides also exhibited quinolone resistance.

The sequencing data for the QRDR of *gyr*A and *par*C (encoding DNA gyrase and DNA topoisomerase IV, respectively) revealed the presence of Ser (TCA) 83 Leu (TTA) and Ser (TCG) 80 Leu (TTG) substitutions in the respective enzymes for all the quinolone-resistant isolates sequenced. No additional amino acid changes were observed in the GyrA polypeptide in these isolates. However, nucleotide sequence changes were observed, GyrA at 71^{th} (GGT to GGG) and 162^{th} (CGA to CGT) and ParC at 109^{th} (CCT to CCA), 124^{th} (AAA to AAG) and 126^{th} (TCG to TCA) in twenty-six clinical isolates (Table 9). These isolates belong to cluster II in the dendrogram with a similarity index of over 90% by REP-PCR (Figure 5). In *A. baumannii*, efflux pump-mediated resistance to antimicrobials is generally associated with the efflux pumps adeABC, AdeIJK, and AbeM[37, 39]. This study also showed a high distribution of *ade*B (99%) and *ade*J (100%) genes in MDR *A. baumannii*.

All of the eight-six *A. baumannii* isolates showed similar or identical AFLP profiles (Figure 2 and 3) and similar band

patterns on REP-PCR profiles (Figure 4 and 5), suggesting that they originated from a common ancestor and clonally spread in the university hospital in Gangwon province. The clonal spread of MDR bacteria is a major factor contributing to the growing problem of antimicrobial resistance. Previous studies have shown the geographically widespread occurrence of MDR *A. baumannii* strains, which are showing genotypically and phenotypically high similarity, suggesting a clonal spread. Nosocomial infections due to MDR *A. baumannii* strains are increasing worldwide. The MDR *A. baumannii* strains are rapidly adapting to the hospital environment, thus, it is becoming difficult to control the outbreaks. Early recognition of imipenem-resistant *A. baumannii* clones is very important to prevent spreading within the hospital environment. Once MDR *A. baumannii* break out, their molecular typing could be helpful in identification of a common source or cross-contamination. The identification of MDR *A. baumannii* is an important step in enabling epidemiological tracing of these strains.

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국문요약

국내 강원도 소재의 한 대학병원에서 분리된 다제내성 *Acinetobacter baumannii*의 유행

다제내성 *Aconetobacter baumannii* 는 전 세계적으로 병원환경에서 분리되는 전염성인자로 큰 문제점을 야기한다. 본 연구에서는 국내의 강원도 소재 한 대학병원에서 분리된 *A. baumannii* 를 대상으로 미생물학적 방법과 내성을 유발하는 유전자를 대상으로 다제내성을 조사하였으며 분리 균주간의 근연도를 확인하였다. 총 86 주의 *A. baumannii* 를 대상으로 carbapenemase 와 metallo-β-lactamase 생성을 시험하기 위하여 변형된 cloverleaf synergy 시험과 EDTA 디스크법을 실시하였다. 항생제 내성을 유래하는 β-lactamases, 16S rRNA methylases, aminoglycoside-modifying enzymes, quinilone resistance determining regions (QRDR)을 확인하기 위하여 PCR 과 염기서열 분석을 실시하였으며, 다제내성 *A. baumannii* 임상분리주간의 유전학적인 근연도를 확인하기 위하여 REP-PCR 과 AFLP 를 실시하였다. 실험결과 모든 다제내성 *A. baumanni* 에서 $bla_{\text{OXA-23-like}}$ 과 $bla_{\text{OXA-51-like}}$ 유전자가 검출되었으며, 모든 임상분리균주에서 $bla_{\text{OXA-23-like}}$ 유전자 상부의 IS*Aba*1 존재가 확인되어 carbapenemase 에 대한 내성을 유도하는 것으로 확인되었다. 또한 *Acinetobacter* derived cephalosporinase 인 bla_{ADC} 유전자 상부에서 IS*Aba*1 이 모든 균주에서 확인되었고, $bla_{\text{ADC-7-like}}$ 유전자가 81 균주에서 발견되었다. aminoglycoside 에 대한 내성을

유발하는 16S rRNA methylase 유전자인 *arm*A 가 70 균주 (81%)에서 확인되었고, phosphotransferase 유전자인 *aac(3)-Ia* 와 *aac(6')-Ib* 를 동시에 가지는 68 균주가 확인되었으며, 이들을 모두 가지는 *A. baumannii* 가 45 주 (52%)로 확인되었다. 또한, quinolone 계 항균제에 내성을 유발하는 DNA gyrase 와 DNA topoisomerase IV 유전자인 *gyr*A 와 *par*C 의 quinilone resistance determining regions (QRDR)에서 유전자의 염기서열 분석한 결과 유전자의 돌연변이로 인하여 Ser (TCA) 83 Leu (TTA)로 Ser (TCG) 80 Leu (TTG)로 각각 아미노산이 치환된 것을 모든 *A. baumannii* 에서 확인하였다. 강원도 소재의 한 대학병원에서 분리된 MDR *A. baumannii* 의 근연도를 확인한 결과, 모든 균주에서 유사하거나 같은 AFLP pattern 과 REP-PCR pattern 이 확인되어 유전학적으로 높은 근연도를 가지는 세균으로 확인되었다. 이 결과는 다제내성을 가지는 *A. baumannii* 가 유사한 유전형을 가지는 개체로부터 전파된 것을 의미하며, 이는 항생제 내성에 대한 문제점의 주원인으로 여겨진다. 본 연구에서 실시한 강원지역에서 분포되어 있는 MDR *A. baumannii* 의 분자생물학적 분석은 항생제내성세균의 역학조사에 중요한 접근방법의 시도로서, 국내의 역학조사에 기초자료로서의 활용이 가능할 것으로 사료된다.

핵심이 되는 말 : 다제내성 *Acinetobacter baumannii*, $bla_{OXA-23-like}$, $bla_{OXA-51-like}$, IS*Aba*I, *arm*A, GyrA, ParC, REP-PCR, AFLP