A Comparison of Genospecies of Clinical Isolates in the *Acinetobacter* spp. Complex Obtained from Hospitalized Patients in Busan, Korea

Gyu-Nam Park^{1,*}, Hye-Sook Kang^{2,**}, Hye-Ran Kim^{3,***}, Bo-Kyung Jung^{1,*},
Do-Hee Kim^{4,*} and Kyung-Soo Chang^{1,†,***}

¹Department of Clinical Laboratory Science, College of Health Sciences,
Catholic University of Pusan, Busan 46252, Korea

²Department of Laboratory Medicine, Maryknoll Medical Center, Busan 48972, Korea

³Department of Clinical Laboratory Science, College of Health and Therapy,
Daegu Haany University, Gyeongsangbuk-Do 38610, Korea

⁴Department of Laboratory Medicine, Busan Veterans Hospital, Busan 46996, Korea

Of the *Acinetobacter* spp., *A. baumannii* (genospecies 2) is the most clinically significant in terms of hospital-acquired infections worldwide. It is difficult to perform *Acinetobacter*-related taxonomy using phenotypic characteristics and routine laboratory methods owing to clusters of closely related species. The ability to accurately identify *Acinetobacter* spp. is clinically important because antimicrobial susceptibility and clinical relevance differs significantly among the different genospecies. Based on the medical importance of pathogenic *Acinetobacter* spp., the distribution and characterization of *Acinetobacter* spp. isolates from 123 clinical samples was determined in the current study using four typically applied bacterial identification methods; partial *rpoB* gene sequencing, amplified rRNA gene restriction analysis (ARDRA) of the intergenic transcribed spacer (ITS) region of the 16~23S rRNA, the VITEK[®] 2 system (an automated microbial identification system) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). *A. baumannii* isolates (74.8%, 92/123) were the most common species, *A. nosocomialis* (10.6%, 13/123) and *A. pittii* isolates (7.5%, 9/123) were second and third most common strains of the *A. calcoaceticus*-*A. baumannii* (ACB) complex, respectively. *A. soli* (5.0%, 6/123) was the most common species of the non-ACB complex. RpoB gene sequencing and ARDRA of the ITS region were demonstrated to lead to more accurate species identification than the other methods of analysis used in this study. These results suggest that the use of *rpoB* genotyping and ARDRA of the ITS region is useful for the species-level identification of *Acinetobacter* isolates.

Key Words: *Acinetobacter* spp., Genomic species, RpoB genotyping, ARDRA of the ITS region, MALDI-TOF MS, Colony morphology

INTRODUCTION

The genus *Acinetobacter* belongs to the order *Pseudomona-*

dales and family *Moraxellaceae*. It comprises Gram-negative, non-motile, oxidase-negative, glucose non-fermenting, strictly aerobic and catalase-positive coccobacilli rods. To date, more than 40 *Acinetobacter* species have been described. Of these,

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* Graduate student, ** Researcher, *** Professor.

† Corresponding author: Kyung-Soo Chang. Department of Clinical Laboratory Science, College of Health Sciences, Catholic University of Pusan, Busan 46252, Korea.

Tel: +82-51-510-0565, Fax: +82-51-510-0568, e-mail: kschang@cup.ac.kr

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A. baumannii is the most clinically significant because it is closely associated with nosocomial infections. It is also most frequently found in clinical samples. *A. baumannii* is associated with a wide range of infections, including bacteraemia, meningitis, pneumonia, urinary tract infection, ventilator associated pneumonia, and wound infections. In recent years, infection due to other *Acinetobacter* spp. that belong to the *A. calcoaceticus*-*A. baumannii* (ACB) complex (including *A. calcoaceticus*, *A. nosocomialis* and *A. pittii*) has raised major concerns worldwide (Vaneechoutte et al., 1995; Cisneros et al., 2002; Lee et al., 2011; Visca et al., 2011; Tien et al., 2012; Almasaudi, 2018).

The rapid and accurate identification of pathogens, and in particular, *Acinetobacter* spp., is a clinically important objective in clinical microbiology. This is primarily because different genospecies have differing biological characteristics and pathogenicity, and this can be relevant when seeking to ensure treatment efficacy. However, identification at the individual species level of *Acinetobacter* is difficult because *Acinetobacter* spp. are very similar phenotypically, while being closely related genetically. As a result, the traditional approaches used to for identify non-*baumannii* *Acinetobacter* species often result in the latter being falsely identified as *A. baumannii*. Therefore, the development of reliable identification methods has been the focus of recent studies. Several genotypic methods have been found to be effective when seeking to differentiate the *Acinetobacter* spp. isolates from one another. Of the molecular techniques employed, sequence analysis of the 16~23S rRNA or *rpoB* genes is widely used in clinical microbiology laboratories. However, these approaches are not sufficiently polymorphic to successfully differentiate between the different *Acinetobacter* species. A number of genomic fingerprinting methods have been proposed for genospecies typing. Amplified rRNA gene restriction analysis (ARDRA) and amplified fragment length polymorphism (AFLP) analysis are examples of useful genomic fingerprinting techniques that are used for clinical identification. However, these methods are laborious, especially when a large group of isolates is under consideration (Koeleman et al., 1998; Cisneros et al., 2002; Lee et al., 2014; Wang et al., 2014).

Matrix-assisted laser desorption/ionization time-of-flight

mass spectrometry (MALDI-TOF MS), a rapid, accurate, and low-cost technique, is successfully utilized to achieve microbial characterization and identification. This technology is applied to identify diverse microorganisms, including bacteria, fungi and viruses in clinical microbiology laboratories (Croxatto et al., 2012; Dingle and Butler-Wu, 2013; Jeong et al., 2016).

The study objective was to compare the identification accuracy of four typically applied bacterial identification methods; partial *rpoB* gene sequencing, ARDRA of the ITS region of the 16~23S rRNA gene, the VITEK® 2 system (an automated microbial identification system), and MALDI-TOF MS. Although the epidemiology of pathogenic *Acinetobacter* spp. has been described, there are few data on pathogenic *Acinetobacter* spp. in Korea. Motivated by on the medical importance of pathogenic *Acinetobacter* spp., an evaluation was performed in the current study of the isolation frequency and characterization of *Acinetobacter* spp. clinical isolates from various clinical samples collected from general hospitals in Busan, Korea, between 2013 and 2015.

MATERIALS AND METHODS

Bacterial isolates

One hundred and twenty-three non-duplicate clinical isolates of *Acinetobacter* strains, which were collected from two general hospitals in Busan, Korea (between March 2013 and February 2015); 68 isolates from sputum (55.3%), 17 isolates from blood (13.8%), 14 isolates from urine (11.4%), 7 isolates bronchial fluid (5.7%), 6 isolates from wound (4.9%), 5 isolates from foley catheter (4.1%), and 3 isolates from pus and ear, respectively (2.4%). All the isolates were identified as *Acinetobacter* spp. using an automated microbial identification system (VITEK®2; bioMerieux, Marcy l'Etoile, France) utilized in conjunction with the VITEK®2 GN-ID card. Reference strain American Type Culture Collection [ATCC] 19606 (*A. baumannii*, genospecies 2) was purchased. The clinical isolates and reference strain were stored at -70°C in a freezer prior to use.

Cultures and isolation

The clinical isolates were recovered by using blood agar

Table 1. Primer pair used in the experiment for *rpoB* gene amplification

Gene	Sequence (5' to 3')	Product size (bp)	Reference
<i>rpoB</i>	Forward: TAC CGT AAA GAC TTG AAA GAA G	380	La Scola et al., 2006
	Reverse : CAA CAC CTT TGT TCC CGT GA		

that containing 5% sheep blood. The agar plates were incubated in the dark at 37°C under a humid conditions for 24~48 hrs. The plates were examined daily to search for the appearance of pure colonies. The colonies were sub-cultured on MacConkey agar for the pure isolation of *Acinetobacter* isolates.

Bacterial genomic DNA extraction of the isolates

The clinical isolates were incubated in a brain-heart infusion (BHI) broth at 37°C for 18 hrs for genotyping. The genomic DNA of each of the 123 *Acinetobacter* clinical isolates was extracted using the Accuprep® Genomic DNA extraction kit (Bioneer, Daejeon, Korea). Bacterial genomic DNA extraction was performed according to the manufacturer's instructions. The extracted genomic DNA was stored at -20°C prior to further molecular analysis.

The *rpoB* gene amplification and sequencing

The *rpoB* gene amplification and sequencing was performed using a specific oligonucleotide primer set for genospecies identification of the clinical isolates (Table 1). PCR was performed using the following optimum conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min (denaturation), 55°C for 30 sec (annealing), and 72°C for 90 sec (extension), and a final extension at 72°C for 7 min. The expected amplification size (of the PCR product) was approximately 380 bp. The PCR products were analyzed by electrophoresis (100 V for 25 min) with 1.5% agarose gel that contained ethidium bromide (EtBr) and visualized under ultraviolet (UV) light. The amplified genomic DNA of the isolates in the gel was purified using the Accuprep® Gel Purification Kit (Bioneer, Daejeon, Korea). The purified PCR products were sequenced via the sequencing service provided by a professional molecular analysis facility (CosmoGENTECH Inc., Seoul, Korea). After sequencing the DNA, the sequences were compared to available

reference data in the Genbank database using Basic Local Alignment Search Tool (BLAST) to determine species identification.

Amplified rRNA gene restriction analysis of the ITS region

ARDRA of the ITS region of the 16~23S rRNA was performed to identify genospecies of the isolates. The sequences of the primers were 5'-TGG CTC AGA TTG AAC GCT GGC GGC-3' (forward) and 5'-TAC CTT GTT ACG ACT TCA CCC CA-3' (reverse).

PCR was performed using the following optimum conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 90 sec (extension), and a final extension at 72°C for 7 min. The length of the amplified product was approximately 1,300 bp. Five restriction enzymes, *AluI* (AGCT), *HhaI* (GCGC), *HaeIII* (GGCC), *MboI* (GATC), and *MSPI* (CCGG) (Enzynomics, Daejeon, Korea), were used for DNA digestion, performed according to the manufacturer's instructions. The restriction fragment pattern of each sample was separated by 2% agarose gel electrophoresis (100 V for 1 hr) in 1 × Tris-borate EDTA buffer and visualized under UV illumination.

Phylogenetic and genetic characterization analysis

The *rpoB* gene sequences obtained from positive *Acinetobacter* spp. samples were analyzed to determine the genospecies. Multiple sequence alignment and sequence comparisons, including reference sequences that corresponding with genospecies in the GenBank database, were made using the Clustal W (ver. 2.1) software.

Phylogenetic analysis was performed using the Maximum-Likelihood (ML) method in MEGA 6.0 software. Nucleotide distances (*p*-distance) and 1,000 replications were employed in the bootstrap analysis.

Fig. 1. Characterization of colonies of *Acinetobacter* spp. clinical isolates on MacConkey agar plates after incubation 24 hrs. (A) Group A isolates appeared as colorless small size smooth colonies with circular margin. (B) Group B isolates appeared as colorless medium size mucoid colonies. (C) Group C isolates appeared as colorless medium size rough colonies.

MALDI-TOF MS analysis

Measurements were performed on VITEK[®] MS instrument equipped with both IVD 2.0 and RUO databases (bio-Merieux, Marcy l'Etoile, France). The isolates were identified on the VITEK[®] MS system in accordance with the manufacturer's instructions. *Escherichia coli* (*E. coli*) ATCC 8739 was used as the calibration strain.

RESULTS

The diversity of *Acinetobacter* spp. colony morphology on MacConkey agar plates

Colony morphology examination is an important method of bacteria identification. MacConkey agar is commonly used for the detection of *Acinetobacter* spp. in clinical cultures. In the current study, the isolates were classified as three different morphotypes according to colony color (pigmentation) and shape (including form, elevation, and margin) on MacConkey agar plates (Fig. 1). One-hundred one isolates (Group A, 82.1%) were observed to be small in size (< 3 mm), smooth (the entire margin), round (circular form), and raised colonies on the MacConkey agar plates following incubation for 24 hrs. Sixteen isolates (Group B, 13.0%) were seen to be of a medium size (3~5 mm), smooth (the entire margin),

shiny, round (circular form), and characterized by slightly mucoid colonies. Six isolates (Group C, 4.9%) were of a medium size, rough, irregular and characterized by flat colonies.

None of the *Acinetobacter* spp. isolates produced any pigment. Non-lactose fermenting colonies (colorless or light lavender color) were observed. The colonies of all isolates were Gram-negative (data not shown).

Phenotypic identification of *Acinetobacter* spp. isolates using the VITEK[®] 2 system

VITEK[®] 2, an automated microbial identification system, is frequently used to rapidly identify pathogens in clinical laboratories around the world. The clinical isolates in the current study were identified using the VITEK[®] 2 system in conjunction with the GN-ID card. All the isolates (n=123, 100.0%) were identified as *A. baumannii* (genospecies 2) (Table 2).

Genotypic identification of *Acinetobacter* spp. isolates using the *rpoB* gene sequencing

Fig. 2 shows the PCR amplification results for the *rpoB* gene. One-hundred and twenty three *Acinetobacter* clinical isolates were analyzed using the *rpoB* gene genotyping (Supplementary data 1). Five *Acinetobacter* spp. were identi-

Fig. 2. ITS region and *rpoB* gene amplification of *Acinetobacter* spp. isolates by PCR. Lanes M; 100 bp molecular marker (Bioneer, Seoul), the others; PCR products of *Acinetobacter* spp. isolates. (A) ITS region was amplified specific fragments of 1,300 bp in *Acinetobacter* spp. isolates. (B) *rpoB* gene was amplified specific fragments of 400 bp in *Acinetobacter* spp. isolates.

Table 2. Detailed results of the identification of 123 *Acinetobacter* clinical isolates obtained using the *rpoB* gene sequencing, ITS region sequencing, the MALDI-TOF MS based VITEK[®]MS, and the VITEK[®]2

Groups	Final Identification (No. of isolates)	Colony type (n)	Identification results				
			Gene sequencing		MALDI-TOF MS (VITEK [®] MS)		VITEK [®] 2 system (n)
			<i>rpoB</i> (n)	ITS (n)	IVD 2.0 (n)	RUO v 4.13 (n)	
ACB complex	<i>A. calcoaceticus</i> (3)	A (3)	<i>A. calcoaceticus</i> / <i>A. pittii</i> (3)	<i>A. calcoaceticus</i> / <i>A. pittii</i> (3)	<i>A. baumannii</i> complex (3)	<i>Acinetobacter</i> spp. (3)	<i>A. baumannii</i> (3)
	<i>A. baumannii</i> (92)	A (76) B (16)	<i>A. baumannii</i> (92)	<i>A. baumannii</i> (92)	<i>A. baumannii</i> complex (92)	<i>A. baumannii</i> (92)	<i>A. baumannii</i> (92)
	<i>A. pittii</i> (9)	A (9)	<i>A. calcoaceticus</i> / <i>A. pittii</i> (9)	<i>A. calcoaceticus</i> / <i>A. pittii</i> (9)	<i>A. baumannii</i> complex (9)	<i>Acinetobacter</i> spp. (9)	<i>A. baumannii</i> (9)
	<i>A. nosocomialis</i> (13)	A (13)	<i>A. nosocomialis</i> (13)	<i>A. nosocomialis</i> / <i>A. calcoaceticus</i> (13)	<i>A. baumannii</i> complex (13)	<i>A. baumannii</i> (13)	<i>A. baumannii</i> (13)
Non-ACB complex	<i>A. soli</i> (6)	C (6)	<i>A. soli</i> (6)	<i>A. soli</i> / <i>A. baumannii</i> (6)	No ID (6)	No ID (6)	<i>A. baumannii</i> (6)

ACB: *A. calcoaceticus*-*A. baumannii* complex, ITS: internal transcribed spacer, MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

fied: *A. baumannii* (genospecies 2), 92 isolates (74.8%); *A. nosocomialis* (genospecies 13TU), 13 isolates (10.6%); *A. calcoaceticus* or *A. pittii* (genospecies 1 or 3), 12 isolates (9.7%, 12 isolates were incorrectly identified as *A. calcoaceticus* or *A. pittii*); and *A. soli*, 6 isolates (4.9%) isolates (Table 2).

The *rpoB* gene sequencing correctly identified 117 isolates (95.1%) belonging to ACB-complex. Only 6 isolates (4.9%) belonging to non-ACB complex.

Phylogenetic analysis using partial *rpoB* gene sequences

The sequences of part of the *rpoB* gene (approximately

Fig. 3. A phylogenetic tree based on *rpoB* gene sequences shows the relationship of the 123 *Acinetobacter* clinical isolates. Numbers on branches indicate percentages of the number of times that the node was supported in 1,000 replicates of bootstrap analysis. Scale bar, 0.02 substitutions per site (Supplementary data 2).

380 bp) from all *Acinetobacter* isolates were aligned and edited, and the following sequences were used as reference strains: *A. calcoaceticus* (GenBank accession number: KJ788852 and KJ788727), *A. baumannii* (GenBank accession number: CP027530 and CP018332), *A. pittii* (GenBank accession number: CP014477 and LC102689), *A. nosocomialis*

(GenBank accession number: LC102686 and KJ789022) and *A. soli* (GenBank accession number: KJ789086 and KJ789092). The *Acinetobacter* clinical isolates were separated into two major groups (group I and II) based on the phylogenetic tree constructed using ML. Group I comprised *A. calcoaceticus*, *A. baumannii*, *A. pittii* and *A. nosocomialis*, in which *Acinetobacter* genospecies 1~3 were observed to be closely grouped. This clustering was supported by high bootstrap value of 99%. Group II comprised *A. soli* (a bootstrap values of 99%) (Fig. 3).

Genotypic identification of *Acinetobacter* spp. isolates using ITS region sequencing

One-hundred and twenty three *Acinetobacter* clinical isolates were analyzed using ITS region genotyping. PCR amplification results for the ITS region are provided in Fig. 2.

As a result of ITS region sequencing, the 5 *Acinetobacter* spp. were as follows: *A. baumannii* (genospecies 2), 92 isolates (74.8%); *A. nosocomialis* or *A. calcoaceticus* (genospecies 13TU or 1), 13 isolates (10.6%, 13 isolates were incorrectly identified as *A. nosocomialis* or *A. calcoaceticus*); *A. calcoaceticus*, *A. pittii* or *A. baumannii* (genospecies 1, 3 or 2), 12 isolates (9.7%, 12 isolates were incorrectly identified as *A. calcoaceticus*, *A. pittii* or *A. baumannii*); and *A. soli* or *A. baumannii*, 6 isolates (4.9%, isolates were incorrectly identified as *A. soli* or *A. baumannii*) isolates (Table 2). Thus, the findings using ITS region sequencing were relatively less accurate than those obtained using *rpoB* gene sequencing.

Molecular analysis of *Acinetobacter* spp. isolates by ARDRA

For genospecies identification of isolates, ARDRA of the ITS region was applied as previously described by Vaneechoutte et al. (Vaneechoutte et al., 1995). ITS region of 16~23S rRNA gene was amplified for a total of 123 isolates belonging to the 5 species of the genus *Acinetobacter* and amplified product was restricted independently with a total of 5 different endonucleases (*AluI*, *HhaI*, *HaeIII*, *MboI*, and *MSPI*). Each enzyme generated up to 5 fragments per isolates.

Fig. 4 shows an overview of different restriction patterns observed. Digestion of the ITS region by *AluI* resulted in 2

Fig. 4. ARDRA patterns of *Acinetobacter* clinical isolates obtained after restriction of amplified ITS region with three different enzymes (*AluI*, *HhaI*, and *MboI*). Lanes M; molecular size marker, 1~10; clinical isolates.

different patterns. Genospecies 2, 3, and 13TU (*A. baumannii*, *A. pittii*, and *A. nosocomialis*) isolates were digested into three different size fragments. Genospecies 1 (*A. calcoaceticus*) isolates were digested into two different size fragment. Digestion by *HhaI* resulted in 2 different patterns. Genospecies 1 and 3 isolates were digested into four different size fragments. Genospecies 2 and 13TU isolates were digested into three different size fragments. Digestion by *MboI* resulted in 2 different patterns. Genospecies 1, 2, 13TU isolates were four different size fragments. Genospecies 3 isolates were digested into four different size fragments. Genospecies 3 isolates were digested into three different size fragments. Combination of the patterns obtained after separate restriction with *AluI*, *HhaI* and *MboI* enabled us to identify the 4 genospecies of *Acinetobacter* spp. isolates. Therefore, four species (1, 2, 3 and 13TU) of *Acinetobacter* isolates were determined by three endonucleases (*AluI*, *HhaI* and *MboI*). However, *A. soli* isolates were not differentiated from other species of *Acinetobacter* isolates, in this study.

After analysis for ARDRA of ITS region, the 5 *Acinetobacter* spp. were identified correctly: *A. baumannii* (geno-

species 2), 92 isolates (74.8%); *A. nosocomialis* (genospecies 13TU), 13 isolates (10.6%); *A. pittii* (genospecies 3), 9 isolates (7.3%); *A. soli*, 6 isolates (4.9%) isolates; and *A. calcoaceticus* (genospecies 1), 3 isolates (2.4%).

MALDI-TOF MS analysis using the VITEK® MS for species identification of *Acinetobacter* isolates

MALDI-TOF MS is a useful method of identifying bacteria and has several benefits. The identification results obtained from the use of two distinct test system (VITEK®-MS: IVD 2.0 and RUO) that were applied to the 123 isolates are summarized in Table 2. One-hundred and seventeen isolates (95.1%) identified as *A. baumannii* complex using the VITEK®-MS IVD 2.0 system. Six isolates (4.9%) were not identified. One-hundred and five isolates (85.4%) were identified as *A. baumannii* using the VITEK®-MS RUO system. Twelve isolates (9.7%) of *Acinetobacter* spp. identified at the genus level. Six isolates (4.9%) were not identified. Each group showed very similar peak profiles, but there were some significant differences between both groups (Fig. 5).

Distribution of *Acinetobacter* spp. isolates by various clinical samples

A. baumannii accounted for 74.8% of the 123 *Acinetobacter* spp. isolates (n=92), and non-*baumannii* *Acinetobacter* spp. accounted for 25.2% (n=31). The distribution of *Acinetobacter* spp. isolates by clinical samples is depicted in Table 3. *A. baumannii* was most frequently isolated in all 123 clinical samples (sputum, blood, urine, foley tip, pus discharge, bronchial fluid, wound and ear discharge). *A. baumannii* only was isolated in pus discharge, the tips of Foley catheters, and wound and bronchial fluid samples (100.0%). All of the *A. soli* isolates (n=6, 100.0%) were isolated in sputum samples.

DISCUSSION

The proportions of *Acinetobacter* spp. isolates has been reported in several previous studies. According to these studies, *A. baumannii* (genospecies 2) is known as the most common species of *Acinetobacter* cause of nosocomial infections. However, the growing number of new infections

Fig. 5. Peak profiles of four representative *Acinetobacter* species isolates generated by MALDI-TOF MS based VITEK MS. The x-axis shows the m/z values and the y-axis indicates of the peaks expressed in arbitrary intensity units. (A) Peak profile of *A. baumannii* isolate. (B) Peak profile of *A. nosocomialis* isolate. (C) Peak profile of *A. pittii* isolate. (D) Peak profile of *A. soli* isolate.

Table 3. The distribution of *Acinetobacter* species isolates by clinical sample type

Isolates Sample type	Acinetobacter species: n (%)					Total (%)
	A. baumannii	A. calcoaceticus	A. nosocomialis	A. pittii	A. soli	
Sputum	45 (66.2)	2 (2.9)	10 (14.7)	5 (7.4)	6 (8.8)	68 (55.3)
Urine	9 (64.3)	1 (7.1)	1 (7.1)	3 (21.4)	–	14 (11.4)
Blood	15 (88.2)	–	1 (5.9)	1 (5.9)	–	17 (13.8)
Bronchial fluid	7 (100.0)	–	–	–	–	7 (5.7)
Foley tip	5 (100.0)	–	–	–	–	5 (4.1)
Wound discharge	6 (100.0)	–	–	–	–	6 (4.9)
Pus discharge	3 (100.0)	–	–	–	–	3 (2.4)
Ear discharge	2 (66.7)	–	1 (33.3)	–	–	3 (2.4)
Total (%)	92 (74.8)	3 (2.4)	13 (10.6)	9 (7.3)	6 (4.9)	123 (100.0)

from non-*baumannii* *Acinetobacter* species is increasingly being recognized, and non-*baumannii* *Acinetobacter* species have been identified as important contributors to nosocomial infection (Lee et al., 2011; Visca et al., 2011; Antunes et al.,

2014). The distribution of *Acinetobacter* spp. isolates, collected from two general hospitals, in Busan, Korea, was evaluated in the current study. *A. baumannii* isolates (74.8%, 92/123) were the most common species, *A. nosocomialis* isolates

(10.6%, 13/123) and *A. pittii* isolates (7.5%, 9/123) were the second and third common strains of ACB-complex, respectively (Table 2). A different *Acinetobacter* species distribution to that reported in other studies was determined in the current research. According to Lee et al., *A. baumannii* (74.9%), *A. nosocomialis* (12.3%) and *A. bereziniae* (3.4%, Genospecies 10; non-ACB complex) were the common species of *Acinetobacter* from 495 clinical isolates (collected from 2005 to 2012) in Korea (Lee et al., 2014). By contrast, *A. bereziniae* was not isolated, and *A. soli* (5.0%, 6/123) was the most common non-ACB complex species, in the present study. (Endo et al., 2014) demonstrated that *A. soli* accounted for a high proportion of *Acinetobacter* isolates (27.1%) that were the cause of bacteremia in a Japanese tertiary hospital (Endo et al., 2014). According to Khosravi et al., *A. baumannii* (66.0%), *A. calcoaceticus* (4.5%) and *Acinetobacter* genospecies 16 (4.0%; non-ACB complex) were the most cited species of *Acinetobacter* identified in 197 clinical isolates (collected from 2011 to 2013) in Iran (Khosravi et al., 2015). These results show that the distribution of *Acinetobacter* spp. varies depending on geographical, institutional and epidemiological differences.

The genus *Acinetobacter* was originally suggested by Brisou and Prevot (1954). Since then, more than 30 *Acinetobacter* species have been described. The identification of *Acinetobacter* at the species level is difficult. The ACB-complex species, in particular, are known to be a common cause of nosocomial infections. *A. baumannii*, *A. calcoaceticus*, *A. pittii* and *A. nosocomialis* are phenotypically very similar and genetically closely related (Vaneechoutte et al., 1995; Lee et al., 2011; Visca et al., 2011; Tien et al., 2012; Almasaudi, 2018). The accurate identification of bacterial pathogens is essential within a clinical laboratory. Various phenotypic and genotypic methods have been developed and validated to ensure the correct identification of different *Acinetobacter* spp. the *rpoB* gene sequencing. ARDRA of the ITS region was demonstrated to have greater accuracy than other methods (i.e., ITS region sequencing, MALDI-TOF MS and VITEK[®]2 system) in species identification in the current research. However, the *A. pittii* and *A. calcoaceticus* isolates (7.5%, 12/123) were not correctly differentiated from one another using *rpoB* gene sequencing and phylo-

genetic analysis. MALDI-TOF MS is presently the predominant method used to identify microorganisms in an increasing number of clinical laboratories. In the current study, the results obtained using the MALDI-TOF MS based VITEK[®]MS system were superior to those obtained using a commercial biochemical approach (i.e., VITEK[®]2). However, 31 isolates (25.2%) could not be identified at the species level. The rate for correct identification at the species level, misidentification, and the lack of identification was 74.8% (92/123), 20.3% (25/123) and 4.9% (6/123), respectively. Only *A. baumannii* isolates were correctly identified at the species level using VITEK[®] MS RUO mode. In the present study, isolates of *A. soli*, which are not included in VITEK[®] MS database, were not identified at all. The system database should be further expanded and optimized with respect to the species that were misidentified. Similarly, rare or recently named species should be added to the database in order to ensure more accurate identification when using MALDI-TOF-based VITEK[®] MS. Jeong et al. (2016) demonstrated that MALDI-TOF MS was a useful method of correctly identifying *Acinetobacter* isolates at the species level following updates to the database (Jeong et al., 2016).

Traditionally, colony formation of microorganisms and its morphological analysis (size, shape, surface and color) with a naked eye have been applied for detection and identification of microbial species (Maeda et al., 2017). In the current study, colonies of 123 *Acinetobacter* spp. isolates were observed on MacConkey agar, and colony based bacterial strain typing was performed. Three types of colonies were identified; (1) small size (< 3 mm), smooth, round shape and raised colonies (Group A), (2) medium size (3~5 mm), smooth, round shape and mucoid colonies (Group B), and (3) medium-large size (5~10 mm), rough, irregular shape and flat colonies (Group C). Group C was identified as *A. soli* only (n=6, 100.0%). Like this result, colony morphological type was related with *Acinetobacter* spp., and considered to be one of useful species screening method in the clinic.

In conclusion, our study demonstrated that the *rpoB* gene sequencing and ARDRA of ITS region of 16~23S rRNA seem to be very useful genotypic methods for the differentiation of *Acinetobacter* spp. at the species level. As a result, 123 *Acinetobacter* spp. isolates were identified correctly: *A.*

baumannii (genospecies 2), 92 isolates (74.8%); *A. nosocomialis* (genospecies 13TU), 13 isolates (10.6%); *A. pittii* (genospecies 3), 9 isolates (7.3%); *A. soli*, 6 isolates (4.9%) isolates; and *A. calcoaceticus* (genospecies 1), 3 isolates (2.4%).

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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