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Comparison of *rpoB* gene sequencing, 16S rRNA gene sequencing, *gyrB* multiplex PCR, and the VITEK2 system for identification of *Acinetobacter* clinical isolates ☆

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ABSTRACT

Since accurate identification of species is necessary for proper treatment of *Acinetobacter* infections, we compared the performances of 4 bacterial identification methods using 167 *Acinetobacter* clinical isolates to identify the best identification method. To secure more non-*baumannii Acinetobacter* (NBA) strains as target strains, we first identified *Acinetobacter baumannii* in a total of 495 *Acinetobacter* clinical isolates identified using the VITEK 2 system. Because 371 of 495 strains were identified as *A. baumannii* using *gyrB* multiplex 1 PCR and $bla_{\text{OXA51-like}}$ PCR, we performed *rpoB* gene sequencing and 16S rRNA gene sequencing on remaining 124 strains belonging to NBA and 52 strains of *A. baumannii*. For identification of *Acinetobacter* at the species level, the accuracy rates of *rpoB* gene sequencing, 16S rRNA gene sequencing, *gyrB* multiplex PCR, and the VITEK 2 were 98.2%, 93.4%, 77.2%, and 35.9%, respectively. The *gyrB* multiplex PCR seems to be very useful for the detection of ACB complex because its concordance rates to the final identification of strains of ACB complex were 100%. Both the *rpoB* gene sequencing and the 16S rRNA gene sequencing may be useful in identifying *Acinetobacter*.

1. Introduction

Acinetobacter species are typical nosocomial pathogens causing infections and high mortality, almost exclusively in compromised hospital patients (Lee et al., 2011a; Sung et al., 2013). *Acinetobacter calcoaceticus*, *Acinetobacter baumannii*, *Acinetobacter* genomic species (AGS) 3 (same as *Acinetobacter pittii*), and AGS sensu Tjernberg and Ursing (AGS 13TU, same as *Acinetobacter nosocomialis*) are closely related genetically and are difficult to distinguish phenotypically using routine laboratory methods. Therefore, it has been proposed that these species be referred to as a group called the *A. calcoaceticus–A. baumannii* complex (ACB complex) (Lee et al., 2011c). However, grouping these species together as a complex may be unsatisfactory and even misleading in clinical settings because they may have different clinical implications (Chiang et al, 2012). Because of the increasing relevance of *A. baumannii* and other genomic species as pathogens, recent research has focused on the development of reliable identification methods (Alvarez-Buylla et al., 2012).

For the identification of *Acinetobacter* species, several genotypic methods have been shown to be adequate, while phenotypic methods have been found to be insufficient (Karah et al., 2011). Among the genotypic methods, 16S rRNA gene sequencing is one of the most commonly used for bacterial identification (Kim and Jang, 2012).

The 16S rRNA gene sequencing can be used in hospitals to identify bacterial species that are difficult to identify using ordinary methods. However, 16S rRNA gene sequencing is not sufficiently polymorphic to distinguish all *Acinetobacter* species (Alvarez-Buylla et al., 2012; Janda and Abbott, 2007).

The RNA polymerase β-subunit (*rpoB*) gene sequences are one of the most useful tools for the identification and taxonomic classification of various bacterial species, including *Acinetobacter* spp. (Alvarez-Buylla et al., 2012; Gundi et al., 2009). Sequence analysis of the *rpoB*

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gene has been found to be a reliable and rapid method for identifying *Acinetobacter* species (Karah et al., 2011; Gundi et al., 2009).

For some bacterial genera, the discriminating power of 16S rRNA gene sequencing is known to be lower than that of *rpoB* gene sequencing (Adékambi et al., 2006; Adékambi et al., 2009; La Scola et al., 2006). However, the accurate identification rates of these 2 methods have been rarely compared in a large number of *Acinetobacter* species isolated from clinical specimens. To date, 16S rRNA gene sequencing has been used more frequently in clinical microbiology laboratories than *rpoB* gene sequencing.

This paper compares the identification accuracies of 4 bacterial identification methods: *rpoB* gene sequencing method, 16S rRNA gene sequencing method, *gyrB* multiplex PCR, and the VITEK 2 identification method. We expect that the identification accuracies of these 4 methods reported here will facilitate selection of the most effective test in various clinical situations.

2. Materials and methods

2.1. Bacterial isolates

A total of 495 clinical isolates identified as *Acinetobacter* species in the Department of Laboratory Medicine in Chosun University Hospital between September 2005 and May 2012 using the VITEK GNI card (bioMérieux, Hazelwood, MO, USA) with the VITEK 2 system (bioMérieux) were randomly selected for this study. We picked out *A. baumannii* first among the 495 strains to secure more non-*baumannii Acinetobacter* (NBA) strains as target strains. We first identified *A. baumannii* by *gyrB* multiplex PCR and $bla_{\text{OXA51-like}}$ PCR tests. Then, we performed detailed molecular identification tests on the remaining strains using *rpoB* gene sequencing and 16S rRNA gene sequencing, etc.

We used 167 strains identified to the species level by detailed molecular identification tests to compare 4 identification methods: *rpoB* gene sequencing, 16S rRNA gene sequencing, *gyrB* multiplex PCR, and the VITEK 2 system.Thereafter, we investigated the distribution of *Acinetobacter* species on the 495 strains identified by *gyrB* multiplex PCR identification tests or detailed molecular identification tests.

We were able to collect patient information from 483 of the 495 patients carrying the *Acinetobacter* species used in this study. The male: female ratio of the 483 patients was 1.9:1. Most patients were middle to old aged. The age distribution of the patients was as follows: 0–9 years, 0.8%; 10–19 years, 1.4%; 20–29 years, 3.7%; 30–39 years, 5.3%; 40–49 years, 13.5%; 50–59 years, 17.0%; 60–69 years, 23.0%; 70–79 years, 24.6%; and 80–89 years, 10.6%. The distribution of departments to which the patients belonged was as follows: neurosurgery, 23.8%; pulmonary and critical care medicine, 19.0%; general surgery, 9.5%; infectious diseases, 8.7%; orthopaedic surgery, 6.2%; chest surgery, 5.6%; nephrology, 4.3%; gastroenterology and hepatology, 4.1%, cardiovascular diseases, 3.5%; emergency medical centre, 2.9%; haematology medical oncology, 3.9%; neurology, 2.5%; urology, 1.0%; plastic surgery, 1.0%; rheumatology, 0.8%; and paediatric and adolescent medicine, 0.8%.The distribution of specimens from which *Acinetobacter* species were isolated was as follows: sputum, 38.5%; whole blood, 18.6%; open pus, 15.3%; closed pus, 6.8%; CSF, 5.8%; catheterized urine, 3.5%; pleural fluid, 2.9%; central venous catheter tip, 2.1%; and others, 6.4%.

2.2. PCR amplification and gene sequencing

Primers used for PCR amplification and sequencing are listed in Table 1 (Chang et al., 2005; Higgins et al., 2010; Higgins et al., 2007; Ragon et al., 2013; La Scola et al., 2006; Miyoshi et al., 2005; Woodford et al., 2006). PCR reactions were carried out using AccuPower® PCR PreMix (Bioneer, Chungwon, Korea) and a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). PCR amplicons were purified for sequencing using the AccuPrep® PCR Purification Kit (Bioneer) according to the protocol of the manufacturer. Sequencing reactions were carried out with the reagents from the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 310 genetic analyser (Applied Biosystems) according to the standard automated sequencer protocol.

Table 1
PCR primers used for species identification and molecular characterization.

Target gene	Primer	Nucleotide sequence 5′-3′	Product size (bp)	References
rpoB gene	rpoB-F	TAY CGY AAA GAY TTG AAA GAA G	350	La Scola et al., 2006
	rpoB-R	CMA CAC CYT TGT TMC CRT GA		
16S rRNA gene	16S-27F	AGA GTT TGA TCM TGG CTC AG	1400	Miyoshi et al., 2005
	16S-1492R	GGT TAC CTT GTT ACG ACT T		Ragon et al., 2013
ITS gene	ITS-F	GTC GTA ACA AGG TAG CCG TA	1500	Chang et al., 2005
	ITS-R	GGG TTY CCC CRT TCR GAA AT		
gyrB gene (*gyrB* multiplex 1 PCR)	Sp2-F	GTT CCT GAT CCG AAA TTC TCG	294	Higgins et al., 2007
	Sp4-F	CAC GCC GTA AGA GTG CAT TA	490	
	Sp4-R	AAC GGA GCT TGT CAG GGT TA		
gyrB gene (*gyrB* multiplex 2 PCR)	Sp1F (D14)	GAC AAC AGT TAT AAG GTT TCA GGT G	428	Higgins et al., 2010
	Sp1R (D19)	CCG CTA TCT GTA TCC GCA GTA		
	Sp3F (D16)	GAT AAC AGC TAT AAA GTT TCA GGT GGT	194	
	Sp3R (D8)	CAA AAA CGT ACA GTT GTA CCA CTG C		
*bla*OXA51-like gene	Oxa-51-like F	TAA TGC TTT GAT CGG CCT TG	353	Woodford et al., 2006
	Oxa-51-like R	TGG ATT GCA CTT CAT CTT GG		

A nucleotide sequence homology search for 16S rRNA gene sequences was performed using an extension of the EzTaxon database (http://www.eztaxon.org) (Chun et al., 2007), which stores 16S rRNA gene sequences of reference strains of validly published names. A nucleotide sequence homology search for *rpoB* gene sequences was performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Sequences were aligned and compared with the published sequences of *Acinetobacter* type strains (Karah et al., 2011; Nemec et al., 2011) (Table 2) by using the Lasergene software package (DNASTAR, Madison, WI, USA).

2.3. Identification of Acinetobacter species

From the 495 *Acinetobacter* test strains, we first identified *A. baumannii* because this species is readily identifiable using simple PCR tests (*gyrB* multiplex 1 PCR and $bla_{\text{OXA51-like}}$ PCR). To identify *A. baumannii*, we analysed 495 *Acinetobacter* clinical isolates by *gyrB* multiplex 1 PCR (Higgins et al., 2007). $bla_{\text{OXA51-like}}$ PCR (Woodford et al., 2006) was performed to verify the *A. baumannii* strains detected by *gyrB* multiplex 1 PCR. The strains verified by positive $bla_{\text{OXA51-like}}$ PCR reactions were identified as *A. baumannii.*

A total of 371 of the 495 strains were identified as *A. baumannii* by simple PCR identification tests. We performed detailed molecular identification using *rpoB* gene sequencing and 16S rRNA gene sequencing on the remaining 124 NBA strains and on 52 strains of *A. baumannii* that were randomly selected from the 371 *A. baumannii* strains identified by simple PCR tests.

We assigned the bacterial species to the test organism in the database that showed the closest match to the tested sequence (Janda and Abbott, 2007). A total of 167 of the 176 strains were identified to the species level. Subsequently, a comparative analysis was conducted