Tropical Biomedicine 28(3): 563–568 (2011)

Research Note

Application of amplified ribosomal DNA restriction analysis in identification of *Acinetobacter baumannii* from a Tertiary Teaching Hospital, Malaysia

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Received 9 January 2011; received in revised form 12 May 2011; accepted 20 May 2011

Abstract. *Acinetobacter baumannii*, genomic species 3 and 13TU are being increasingly reported as the most important *Acinetobacter* species that cause infections in hospitalized patients. These *Acinetobacter* species are grouped in the *Acinetobacter calcoaceticus-Acinetobacter baumannii (Acb)* complex. Differentiation of the species in the *Acb*-complex is limited by phenotypic methods. Therefore, in this study, amplified ribosomal DNA restriction analysis (ARDRA) was applied to confirm the identity *A. baumannii* strains as well as to differentiate between the subspecies. One hundred and eighty-five strains from Intensive Care Unit, Universiti Malaya Medical Center (UMMC) were successfully identified as *A. baumannii* by ARDRA. *Acinetobacter* genomic species 13TU and 15TU were identified in 3 and 1 strains, respectively. ARDRA provides an accurate, rapid and definitive approach towards the identification of the species level in the genus *Acinetobacter*. This paper reports the first application ARDRA in genospecies identification of *Acinetobacter* in Malaysia.

INTRODUCTION

Bacteria of the genus *Acinetobacter* are gram negative coccobacilli, non-fermenting, non-motile, strictly aerobic, oxidase-negative and can grow well on common complex media (Baumann, 1968; Rossau *et al.*, 1991). To date, 32 species (21 named species; 11 genomic species) have been described within the genus *Acinetobacter* (Dijkshoorn *et al.*, 2007), which are widely distributed in nature and can be isolated from water, soil, and even human skin. Some of these genospecies are capable of causing nosocomial infections such as bacteremia, meningitis, respiratory and urinary tract infections particularly in immuno-compromised patients (Bergogne-Berezin & Towner, 1996). Recently, *Acinetobacter baumannii* has been reported as an important nosocomial pathogen causing infections in the intensive care units in hospitals worldwide (Hanlon, 2005; Perez *et al.*, 2007).

Identification of *Acinetobacter* spp. based on phenotypic methods have limited capacity and are not accurate for differentiation of the genus to the genospecies level (Gerner-Smidt *et al.*, 1991; Bernards *et al.*, 1995, 1996). Few methods have been developed to overcome the limitation of phenotypic method and these include tRNA spacer fingerprinting (Ehrenstein *et al.*, 1996), 16S-23S spacer regions (Nowak & Kur, 1995), sequence variations in *gyr*B genes

(Yamamoto & Harayama, 1996) and *rec*A-RFLP (Jawad *et al.*, 1998). Among these, amplified ribosomal DNA restriction analysis (ARDRA) was reported to be the useful method to give definitive subtyping results (Dijkshoorn *et al.*, 1998; Jawad *et al.*, 1998; Shin *et al.*, 2004). ARDRA involves restriction digestion of the amplified 16S rDNA with at least 5 different enzymes. The restriction patterns are then visualised via agarose gel electrophoresis. The grouping of the genospecies can be determined based on the published characteristic banding patterns (Dijkshoorn *et al.*, 1998). The objective of this study was to confirm the identification and subtyping *Acinetobacter* strains by ARDRA.

A total of 171 *A. baumannii* strains isolated from tracheal secretions (n=86), tracheal aspirate (n=2), sputum (n=6), swab (n=22), catheter tips (n=20), blood (n=11), body fluids (n=15), nasal (n=2), urine (n=3) and tissues (n=3) over a period of 2006 to May 2009 from Intensive Care Units (ICU), Universiti Malaya Medical Center (UMMC) were available for analysis. Another 18 *A. baumannii* strains from the ICU environment (beds, tables, buckets, washbasins, ventilators, mattress, washing sinks, mop and hands of healthcare workers) in 2006 were also included for analysis. All the strains were initially identified as *A. baumannii* by biochemical tests. These strains were sub-cultured on Brain Heart Infusion agar (BHI) to check the purity of the strains for further genospecies identification by ARDRA.

A single bacterial colony on BHI agar plate was picked and suspended in a 0.5 ml microfuge tube containing 50 µl of sterile deionized water. The cell suspensions were boiled for 5 min at 99°C and were kept in ice immediately for 10 min. The cell debris was spun down at 13,000 rpm for 2 min and approximately 5 µl (100 ng) was used for PCR amplification of 16S rDNA as described previously by Vaneechoutte *et al.* (1995) with minor modifications to confirm the identification of the *A. baumannii*. Four different sources of *Taq* DNA Polymerases (GoTaq® DNA Polymerase, Promega, Madigan, USA; *i-Taq*™, iNtRON Biotechnology, Korea, *TaKaRa Ex Taq*™ Takara, Shiga, Japan and HotStarTaq, Qiagen, USA) were initially tested for PCR amplification of 1500 bp ribosomal DNA. The PCR reaction was carried out in a final volume of 25 µl 1x PCR buffer, 1.5 mM $MgCl_2$, 100 µM of each dNTP, 0.2 µM (each) primer and 0.5 U of *Taq* DNA polymerase of each tested *Taq* polymerase, respectively, with the exception of HotStarTaq DNA polymerase where 12.5 µl of HotStarTaq Master Mix (2.5 U of HotStarTaq DNA polymerase, 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTPs) (QIAGEN, USA) was used. The primer sequences for 16S rDNA were 5' TGGCTCAGATTGAACGCTGGCGGC 3' and 5' TACCTTGTTACGACTTCACCCCA 3'. The PCR conditions comprised of an initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 40 s, 50°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 7 min. Annealing temperature at 60°C was used for HotStarTaq DNA polymerase. The PCR products were analysed by electrophoresis on a 1.5% agarose gel (Promega, Madison, USA) with 100 bp ladder as a molecular size marker. The gel was stained with ethidium bromide (0.5 mg/ml) for 5 min and visualized under UV light by using Gel Doc™ XR imaging system (Bio-Rad, USA).

Approximately 5 µl of the amplified PCR product was digested with 2U restriction enzymes *Alu*I (AGCT), *Cfo*I (GCGC), *Mbo*I (GATC), *Msp*I (CCGG) and *Rsa*I (GTAC) in 20 µl total volume of commercially supplied restriction buffers (Promega, Madison, USA), respectively and incubated at 37°C for 4 hours. The fragments obtained by digestion with each enzyme were electrophoretically separated in 1.5% agarose gels. A 100 bp ladder (Promega) was used as molecular size marker. Then the gel was stained with ethidium bromide (0.5 mg/ml) for 5 min, destained and then visualized and photographed under UV light using Gel DocTM XR imaging system (Bio-Rad, USA). Species identification was done by comparing the profiles consisting of the combination of restriction patterns generated with the different enzymes with reference to the scheme of Vaneechoutte *et al.* (1995)

(http://allserv.rug.ac.be/_mvaneech/ARDRA/ Acinetobacter.html).

Initially, we used four different sources of *Taq* DNA Polymerases that is GoTaq® DNA Polymerase (Promega, Madigan, USA), *i-Taq*™ (iNtRON Biotechnology, Korea), *TaKaRa Ex Taq*™ (Takara, Shiga, Japan) and HotStarTaq (Qiagen, USA) to amplify the ribosomal DNA. However, the expected amplicon of approximately 1500 bp was only obtained with HotStarTaq DNA polymerase. The other sources of *Taq* polymerases generated unspecific DNA fragments (data not shown). Subsequent PCR amplification of 16S rDNA from the rest of the strains was carried out with the HotStar Taq and gave the expected size of 1500 bp amplicon (Figure 1). After digestion with respective restriction enzymes *Alu*I, *Cfo*I, *Mbo*I, *Msp*I and *Rsa*I, the combination of the restriction patterns obtained gave identification to the species level of the strains (Figure 2, Table 1). Among the 189 (171 clinical and 18 environmental) strains that were initially identified as *A. baumannii*, 185 (97.9%) strains (170 clinical; 15 environmental) were confirmed as *A. baumannii*, 3 (1.6%) strains (1 clinical; 2 environmental) as genospecies 13TU and one environmental strain as genospecies 15TU.

A rapid and accurate identification of bacterial strains is important especially when it involves outbreak cases in hospitals. Bacteria of the genus *Acinetobacter* have been reported worldwide as important nosocomial pathogens that cause severe infections in hospitalized patients (Bergogne-Berezin & Towner, 1996; Levin *et al.*, 1996; Dalla-Costa *et al.*, 2003). *Acinetobacter baumannii*, in particular, is the most common species of *Acinetobacter* that cause infections in immunocompromised patients admitted to ICU. A majority of the strains of this bacterium are able to gain resistance against most available antimicrobial agents and can survive in hospital environments for long periods (Webster *et al.*, 2000).

Phenotypic methods to identify the species of the genus *Acinetobacter* require specific media and are time consuming, requiring a few days of incubation. In addition, this method is inadequate and inaccurate for differentiating between members of the *Acinetobacter calcoaceticus-Acinetobacter baumannii (Acb)* complex which comprise of *A. calcoaceticus* (genospecies 1), *A. baumannii* (genospecies 2), *A.* genospecies 3, and 13TU (Gerner-Smidt *et al.*, 1991; Bergogne-Berezin & Towner, 1996).

Although $bla_{\mathrm{OXA\text{-}51}}$ gene, an intrinsic gene harboured by *A. baumannii*, was reportedly used to identify *A. baumannii* strains (Turton *et al.*, 2006), ARDRA is able to give more information to identify other species of *Acinetobacter* causing infections in the hospitals. Recently, Chang *et al.* (2006)

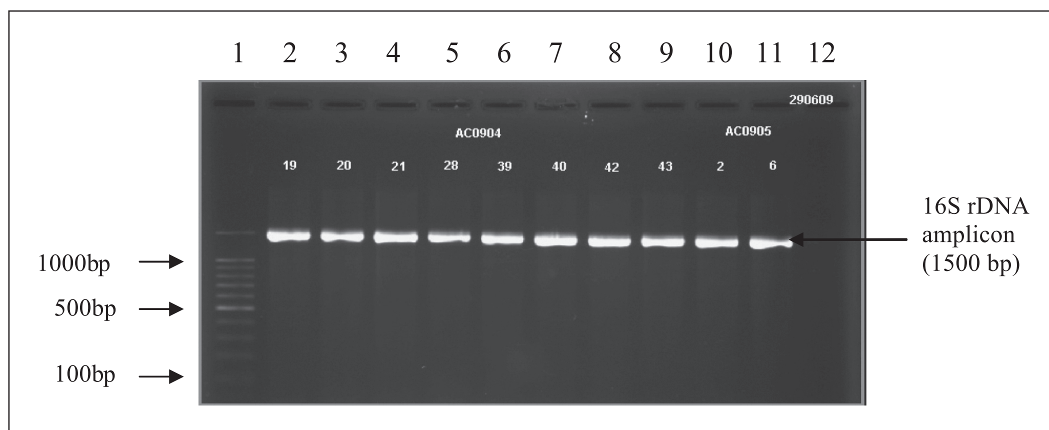

Figure 1. Representative gel of 1500 bp amplicon of the 16S rDNA gene amplified in the ARDRA-PCR. Lane 1: 100bp marker; lane 2-lane 11: *A. baumannii* strains and lane 12: Negative control

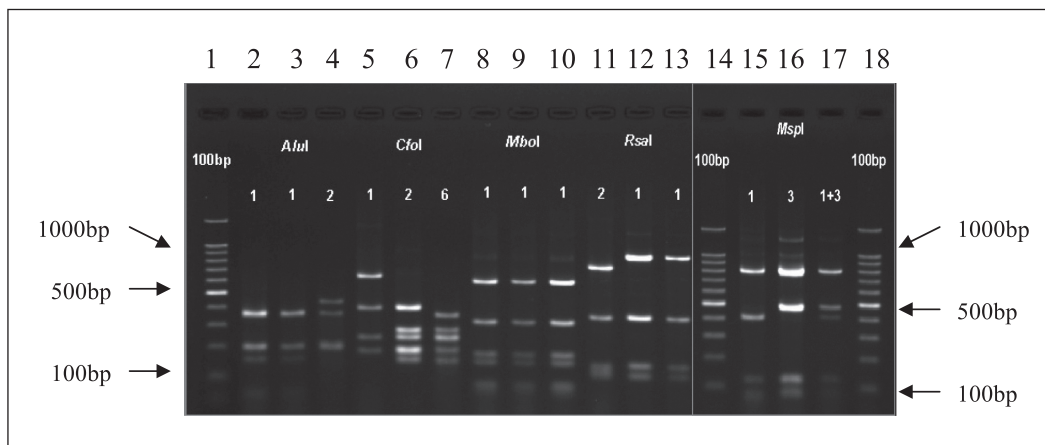

Figure 2. A composite of restriction patterns obtained after digestion with *Alu*I, *Cfo*I, *Mbo*I, *Msp*I and *Rsa*I for an amplified 1500bp of the 16S rDNA gene in Malaysian *A baumanii* isolates. Numbers on each lane refer to the ARDRA pattern of each restriction enzyme (see Table 1). The interpretation was done based on the scheme of Vaneechoutte *et al.* (1995). Lane 1, 14 and 18: 100bp DNA ladder as the molecular size marker (Promega, USA); lane 2, 5, 8, 11 and 15: AC/0612-17 (*A. baumannii*); lane 3, 6, 9, 12 and 16: ACIBA 2006-66 (*Acinetobacter* 13TU); lane 4, 7, 10, 13 and 17: ACIBA 2006-58 (*Acinetobacter* 15TU)

Table 1. Identification and differentiation of *Acinetobacter* strains, in a tertiary teaching hospital, Malaysia, based on ARDRA profiles

Genospecies	ARDRA patterns with restriction enzymes					Number of tested strains
	*Alu*I	*Cfo*I	*Mbo*I	*Rsa*I	*Msp*I	
Genospecies 1 (*A. calcoaceticus*)	2	2	1	1	3	0
Genospecies 2 (*A. baumannii*)	1	1	1	2	1	81
	1	1	1	2	3	89
	1	1	1	2	1+3	15
Genospecies 3	1	2	3	1	3	0
Genospecies 13TU	1	2	1	1	1	1
	1	2	1	1	3	2
	1	2	1	1	1+3	0
Genospecies 15TU	2	6	1	1	3	1

have reported the use of ITS sequencing for identification of the *A. calcoaceticus-A baumannii* complex. However this method is only feasible if a DNA sequencing facility is cheaply and readily available. In the absence of such a capital intensive facility, the ARDRA method, which has been validated by various laboratories, is the best alternative since PCR machines are easily accessible in most clinical microbiology laboratories (Chang *et al.*, 2006).

In conclusion, we have successfully demonstrated the use of ARDRA to identify, differentiate and confirm strains of *A. baumannii*. Different sources of *Taq* DNA polymerase have effects on the amplification of the 16S rDNA. ARDRA was also economical and gave rapid results for identification of the *Acinetobacter* species. This is the first report in Malaysia of differentiation of *Acinetobacter* using ARDRA.

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