Genomic species identification of Acinetobacter of clinical isolates by 16S rDNA sequencing

S Misbah, H Hassan, M Y Yusof, Y A Hanifah, S AbuBakar

ABSTRACT

Introduction: This study aims to identify *Acinetobacter* of clinical isolates from the University of Malaya Medical Centre (UMMC), Kuala Lumpur, to the species level by 16S rDNA sequencing.

Methods: 12 representative *Acinetobacter* isolates of the UMMC inpatients were randomly picked and used for the study. The 16S rDNA sequences were determined and phylogenetic relationships to all known *Acinetobacter* species were established.

Results: Based on the 16S rDNA sequences, all the UMMC isolates were identified as *Acinetobacter baumannii*. The isolates shared a common ancestral lineage with the prototypes *Acinetobacter baumannii* DSM30007 and DSM30008 with 99-100 percent sequence similarities. The isolates could be differentiated into two groups by a single nucleotide difference (thymine-cytosine) within the 16S rRNA sequence. Three different genotypes, 1, 3 and 4, were recognised using REP-PCR.

Conclusion: The previously uncharacterised *Acinetobacter* isolates from the UMMC were identified by their 16S rDNA sequences as *Acinetobacter baumannii*. The isolates were distinguished into at least three different genotypes by REP-PCR genotyping. These findings confirmed for the first time the presence of *Acinetobacter baumannii* of different genotypes among patients at UMMC.

Keywords: *Acinetobacter*, genomic species identification, genotyping, 16S ribosomal RNA

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INTRODUCTION

The genus *Acinetobacter* belongs to the family Moraxellaceae, within the gamma subdivision of proteobacteria. They are gram-negative, strictly aerobic, non-motile coccobacilli that are oxidase-negative and catalase-positive. The bacteria are ubiquitous and can be isolated from soil and water[1]. They are commonly associated with skin colonisation of hospitalised patients and have been associated with serious infections. Over the recent decades, the *Acinetobacter* species (spp.) has increasingly been implicated in outbreaks of nosocomial infections causing pneumonia, bacteraemia, urinary tract infections, wound infections and meningitis worldwide[2,3].

Presently, at least 24 genomic species (DNA group) have been described within the genus *Acinetobacter*[4]. Strains belonging to some of the genomic species are very similar that identification by phenotypic characterisation has always been difficult[5]. It has been reported that most of the isolates of clinical origins are closely related to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* (*Acb*) complex, which comprised of *A. calcoaceticus*, *A. baumannii*, genospecies 3, TU13, "close to TU13" and "between 1 and 3"[6]. With the exception of the genomic species 1, which has been regarded as an environmental species, each genomic species within the *Acb* complex has been shown to be associated with outbreaks of nosocomial infections. *A. baumannii,* in particular, is commonly isolated in hospital outbreaks and has been attributed to cause approximately 45.3% mortality in patients with bacteraemia[7]. A number of *A. baumannii* strains have also developed resistance towards multiple antibiotics, leading to its persistent presence in the hospital environment[8]. These antibiotic-resistant strains pose a significant threat to hospital patients, especially those with immune-compromised conditions.

Presently, bacterial species identification using the 16S rDNA-based method is the most widely accepted method as large public-domain sequence databases are available for comparison[9,10]. The bacteria, however, are typed molecularly using various methods, including pulsed-field gel electrophoresis[11], random amplified polymorphic DNA[12], amplified fragment length polymorphism[13],

Department of Medical Microbiology
Faculty of Medicine
University of Malaya
50603 Kuala Lumpur
Malaysia

S Misbah, BSc
Postgraduate Student

H Hassan, MBBS
Associate Professor

M Y Yusof, MBBS
Associate Professor

Y A Hanifah, MBBS
Associate Professor

S AbuBakar, PhD
Professor

Correspondence to:
Prof Sazaly AbuBakar
Tel: (60) 3 7967 5756
Fax: (60) 3 7967 5757
Email: sazaly@um.edu.my, sazaly@ummc.edu.my

Table I. *Acinetobacter* spp. of clinical isolates from the UMMC used in the study.

Strain no.	Source	Year of collection	Genotype*
M3-66	NA	1987	1
M26-29	Skin	1996	1
M27-53	Urine	1996	1
M30-57	Skin	1997	1
YS074	Trachea	1998	1
M2-82	NA	1987	4
M26-23	Skin	1996	4
M27-67	Skin	1996	4
M28-47	Skin	1996	4
YS304	Throat	1998	4
M26-11	Urine	1996	3
M28-7	Skin	1996	3

NA = not applicable.

* Characterised by REP-PCR DNA fingerprinting[16].

Table II. Oligonucleotide primers used for amplification and sequencing of the *Acinetobacter* spp. 16S rRNA gene.

Primers	Sequence (5' to 3')	Position*
27F'	AGAGTTTGATCCTGGCTCAG	8 - 27
780R'	TACCAGGGTATCTAATCCTGTT	780 - 801
529F'	GTGCCAGCMGCCGCGG	514 - 529
1099R'	GGGTTGCGCTCGTTG	1099 - 1113
925F'	AAACTYAAAKGAATTGACGG	906 - 925
1491R'	ACGGCTACCTTGTTACGACTT	1491 - 1511

All primer sequences are presented in 5' to 3' orientation. Primers were derived from the 16S rRNA sequences of *E. coli*[17,18].

* Position numbering is based on the *E. coli* sequences.

amplified ribosomal DNA restriction analysis[14] and repetitive extragenic palindromic (REP)-PCR[15]. In the present study, the 16S rDNA sequences of the previously-unidentified representative of the predominant *Acinetobacter* isolates of the University of Malaya Medical Centre (UMMC) were determined.

METHODS

A total of 12 representatives of the predominant *Acinetobacter* clinical isolates collected from UMMC were used. These isolates were initially identified by the standard laboratory biochemical tests and typed by REP-PCR as previously described (Table I)[16].

Acinetobacter isolates were cultured on nutrient agar plates (Oxoid Ltd, Hampshire, UK) at 37ºC overnight. Following the incubation, bacterial colonies were picked with sterile wooden toothpicks, suspended in sterile milli-Q water (Millipore, Molsheim, France) and boiled for 5 minutes. The suspension was then centrifuged at 12,000 x g for 10 min and the resulting supernatant containing the bacterial DNA (50-100 ng) was used as template for the polymerase chain reaction (PCR) amplification. Amplification of the 16S ribosomal DNA was performed in a DNA thermal cycler, PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, USA) with the following cycling programme: Initial denaturation at 95ºC for 3 min, and 30 cycles of denaturation at 95ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 1 min and a final extension at 72ºC for 5 min.

The bacterial DNA was amplified using three sets of primers (Table II)[17,18], synthesised by Research Biolabs Pte Ltd (Singapore). All reactions were performed in 25 μl volumes, containing 15 pmol of each primer, PCR nucleotide mix (dNTPs), Mg free buffer, $MgCl_2$ and Taq DNA polymerase (Promega, Madison, USA). The amplified DNA fragments were gel-purified using QIAquick™ Gel Extraction Kit (250) (Qiagen, Hilden, Germany) and sequenced by Macrogen Inc (Seoul, Korea) using an ABI3730 XL DNA Analyser (Applied Biosystems, Renton, USA). Sequence results from each amplified fragment were aligned and assembled into contigs to obtain a complete 16S rDNA consensus sequence using Sequencher™ ver. 4.0.5 (Gene Codes Corporation, Ann Arbor, USA).

The 16S rDNA sequences of the isolates along with sequences of all the known *Acinetobacter* genospecies prototype strains retrieved from the GenBank were aligned using ClustalX (version 1.81) and manually optimised using GeneDoc (version 2.6). Phylogenetic tree was constructed using the neighbour-joining algorithm and the resulting tree was displayed using TreeView (version 1.6.6). Bootstrapping was performed (1,000 replicates) to assess the confidence values of the clusters formed. Identification to the genomic species level was defined as a 16S rDNA sequence similarity above 99%[19] with the prototype strains sequence. Only prototype strains with approximately 1,500 nucleotide sequences were selected for comparisons. Four prototype strains, ATCC19606^T, KF714, DSM30007 and DSM30008, belonging to the *A. baumannii* genospecies 2, were included.

RESULTS

The 16S rDNA genome region consisting of approximately 1,500 nucleotides was amplified using three primer pairs. Amplification using the first set of primers generated a single DNA fragment

of approximately 750 bp (Fig. 1a). Subsequent amplification using the other two primer pairs generated distinctive DNA bands at about 580 bp (Figs. 1b, c). From these amplified DNA fragments, a total of 1,453 consensus nucleotides representing the 16S rRNA genes were obtained. Two groups of isolates differing at only nucleotide position 43 of the gene sequence were identified (Fig. 2). The isolates with thymine (T) at this position belong to the REP-PCR genotype 1, whereas isolates with cytosine (C) comprised isolates of both the REP-PCR genotypes 3 and 4. There were no 16S rDNA sequence differences within the later group of isolates.

The phylogenetic tree constructed using at least 92% of the nucleotide sequence length which corresponded to 1,343 nucleotides (the reference prototype strain sequences available in the GenBank are short by about 50 nucleotides at both ends) showed that all the UMMC isolates grouped into the *A. baumannii* genomic species 2 lineages, represented by the prototype *A. baumannii* strains; DSM30007, DSM30008, KF714 and ATCC 19606^T (Fig. 3). A 100% sequence similarity with *A. baumannii* prototype strains DSM30007 and DSM30008 was noted, as well as 99.9% and 99.8% similarity to *A. baumannii* prototype strain KF714 and ATCC 19606^T, respectively. These findings were in agreement with results previously obtained by REP-PCR fingerprinting that placed all the UMMC isolates in the same lineage as *A. baumannii*[16]. Hence, it was confirmed here that all the UMMC *Acinetobacter* isolates are *A. baumannii*.

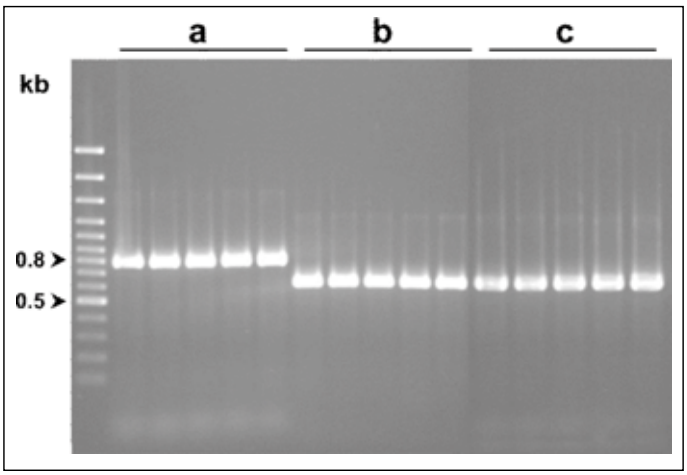

Fig. 1 Amplification of *Acinetobacter* spp. of clinical isolates 16S rRNA gene. Three different PCR primer sets were used to amplify ~1,500 bp of the *Acinetobacter* spp. 16S rRNA gene. (a) DNA fragments of approximately 750 bp were amplified using the first primer pairs. (b,c) Amplification using the other two primer sets both demonstrated amplification of approximately 580 bp DNA fragments, respectively. Only representative five isolates are shown. The first lane on the left is the 100 bp plus DNA marker.

DISCUSSION

Accurate identification and typing of bacterial isolates are essential, particularly when determining strains involved in hospital outbreaks. Presently, molecular methods including DNA-DNA hybridisation and 16S rDNA gene sequencing have been widely adopted as a means of identification of bacteria, and methods, such as REP-PCR, pulsed-field gel electrophoresis, random amplified polymorphic DNA and amplified fragment length polymorphism, are used for typing of likely-related strains. At UMMC, the present practice was to lump together all the *Acinetobacter* strains identified as *Acb* complex using the standard biochemical tests. No attempt so far has been made to identify them to the species level.

Previous typing of 109 *Acinetobacter* spp. at UMMC, however, demonstrated the presence of three predominant fingerprint profiles, identified as genotype 1, 3 and 4. These genotypes were found to be associated with the unique profile of several *A. baumannii* reference isolates. Identification of the UMMC isolates to the species level, however,

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                *         20         *         40         *         60         *         80
M3-66  : GCTTAACACATGCAAGTCGAGCGGGGGAAGGTAGCTTGCTACTGGACCTAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGC
M26-29 : ................................................................................
M27-53 : ................................................................................
M30-57 : ................................................................................
YS074  : ................................................................................
M2-82  : ........................................C.......................................
M26-23 : ........................................C.......................................
M27-67 : ........................................C.......................................
M28-47 : ........................................C.......................................
YS304  : ........................................C.......................................
M26-11 : ........................................C.......................................
M28-7  : ........................................C.......................................
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Fig. 2 Alignment of the UMMC *Acinetobacter* spp. isolates 16S rDNA sequence. Consensus sequence obtained following assembly of the contigs was aligned. A single nucleotide difference at position 43 (arrow) among the UMMC isolates was noted.

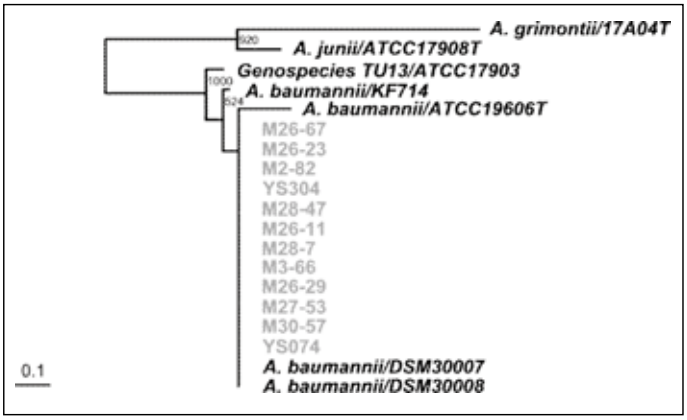

Fig. 3 Identification of the UMMC *Acinetobacter* spp. clinical isolates using the 16S rDNA sequences. The 16S rDNA sequences were aligned and used to construct the neighbour-joining phylogenetic tree. The scale bar indicates the genetic distance and the numbers shown next to each node indicate the bootstrap values from 1,000 replications.

was not made[16]. In the present investigation, 12 representative *Acinetobacter* clinical isolates belonging to the previously-identified REP-genotypes[16] were identified by sequencing the bacteria 16S rRNA genes.

The very high 16S rDNA sequence similarity observed among the different REP-PCR genotypes reflect the extremely slow rate of base substitutions within the 16S rDNA gene and a very rare occurrence of base substitution among the closely-related bacterial strains[20,21], highlighting its usefulness for bacterial species identification. In addition, results from the REP-PCR typing demonstrate the possibility that there were multiple sources of *A. baumannii* at UMMC. This supports the earlier concern that rigorous monitoring and molecular surveillance are necessary to ensure rapid identification and containment of any potential nosocomial outbreak caused by any one of the predominant *A. baumannii* genotypes at the UMMC[16].

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