Value of phenotypic and genotypic identification of *Acinetobacter baumannii* isolates from two hospitals in Jordan

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Abstract

A total of 12 *Acinetobacter baumannii* isolates have been recovered from hospitalized patients at two hospitals in Jordan over two different periods of time (2006 and 2008). Phenotypic and biochemical characterization with antibiotic susceptibility testing indicated that all isolates were belonging to *A. baumannii*. A high degree of conservation of both the 16S-23S rRNA gene intergenic spacer (ITS) length and the ITS sequence was observed among the isolates, and their identities were further confirmed by amplified ribosomal DNA gene restriction analysis (ARDRA). The application of ITS sequence-based identification and ARDRA provide promising tools for the elucidation of the clinical significance of genospecies identification of *A. baumannii*.

Keywords: *Acinetobacter baumannii*, genotyping, multi-drug resistance.

Introduction

Studies from different parts of the world have demonstrated that *Acinetobacter baumannii* is a common opportunistic nosocomial pathogen, with increasingly global prevalence [1]. *A. baumannii* has been found to be responsible for various infections including nosocomial pneumonia, blood infection, urinary tract infection and surgical wound infection especially in patients hospitalized in intensive care unit (ICU) [1-5]. The epidemiology and control of *A. baumannii* infections in hospitals is a complex issue, since this organism has a great capacity to survive in low-moisture environment coupled with its ability to develop rapidly resistance to antimicrobial agents posing particular risk to hospitalized patients [4-5].

A study by Shehabi and Baadran (1996) revealed that few types of multidrug-resistant Gram-negative bacteria (*A. baumanii, Pseudomonas aerugino-*

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sa and *klebsiella pneumoniae*) were commonly associated with Jordanian ICU patients during 1993 [6]. Since then there was increased incidence of *A.baumannii* infections and colonization in Jordanian hospitals [5,7]. In addition, clinical laboratories in Jordan, as other clinical laboratories in other parts of the world, depend mainly upon traditional phenotypic identification methods that relay on biochemical pathways and carbon source utilization [5-7]. Investigators over the years have tried a variety of laboratory improvement tests for identification *A.baumannii* through appling phenotypic and genotypic techniques [8]. Of such efforts was the use of gas chromatographic analysis [9]. Microlog system, comprising a microtiter plate that tests for the ability of a microorganism to utilize different carbon sources [10], VITEK-2 ID-GNB card (bioMérieux, Hazelwood, MO), and Sherlock microbial identification system which is a fully automated gas chromatographic analytical system that identifies bacteria based on their unique fatty acid profiles [11]. A phenotypic identification of *Acinetobacter* to the species level was always considered presumptive, and not so accurate since it is useful only for rapid diagnosis of infections [12,13] .

Taxonomy of the genus *Acinetobacter* has revealed 23 validly named species (http://www.bacterio.cict.fr/) and a number of DNA-DNA hybridization groups (genomic species) with provisional designations [14]. However, the identification of *Acinetobacter* at species level is still erratic and quite variable that hindered progress understanding the pathogenic role of *Acinetobacter* [15]. Thus, precise identification of *Acinetobacter* species would be significant in epidemiology, and infection control policies [16]. Since 1986, the taxonomy of the genus *Acinetobacter* was revised, molecular methods provided the necessary tools to identify *Acinetobacter* at the species level. Detailed studies of the epidemiology of the different members of this genus became possible using genotyping methods [17]. Molecular methods that have been developed

and validated for identification of *Acinetobacters* include amplified 16S rRNA gene restriction analysis (ARDRA) [18], high-resolution fingerprint analysis by amplified fragment length polymorphism (AFLP) [19], ribotyping [20], tRNA spacer fingerprinting [21], restriction analysis of the 16S-23S rRNA intergenic spacer sequences [22], sequence analysis of the 16S-23S rRNA gene spacer region [12], and sequencing of the *rpoB* (RNA polymerase β-subunit) gene and its flanking spacers [23]. Currently, ARDRA and AFLP are the most widely used validated reference methods for species identification of *Acinetobacters* [18,19]

The tremendous increase in sequencing, lead to partial or nearly complete sequence analysis of the 16S rRNA gene for *Acinetobacter* classification [24,25]. In addition, the 16S and 23S rRNA genes intergenic spacer (ITS) has been suggested to be a good candidate for bacterial species identification [26], since these regions have degrees of low intraspecies variation and high degrees of interspecies divergence [27,28]. Therefore, in the present study we have applied 16S-23S rRNA gene intergenic spacer (ITS) sequencing technique to analyze the genotyping of clinical isolates of *A.baumannii* that were collected from two different hospitals in Jordan, and to correlate their genotypes with phenotypic characters .

Materials and Methods

Bacterial isolates

A total of 12 isolates *of A. baumannii* were recovered from 12 clinical samples and included in this study. Seven isolates were recovered from ICU patients of the King Hussein Cancer Center (KHCC) in 2006. The rest 5 isolates were recovered from ICU patients of The Jordan University Hospital in 2008. The clinical samples were isolated from 12 patients with different types of infections at two different periods of time. Pure clinical isolates, identified as *A. baumannii* in the microbiology laboratory of the

2 hospitals were supplied for conducting this research.

The isolates were subjected to repeat phenotypic identification using a simplified scheme for *Acinetobacter spp* [29]. In addition to colony morphology, Gram staining reaction and microscopic characterization, the scheme included growth at 37°C, 41°C, and 44°C, glucose oxidation, gelatin hydrolysis and Triple sugar iron (TSI) test [30]. Biochemical reactions using API 20 NE system (bioMérieuxVitek, Marcy l'Etoile, France) were also used .

Standard control strains of *Pseudomonas aeuroginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922 strains) were used simultaneously with tested clinical isolates. These controls were used for the purposes of justifying results obtained in several tests including antibiotic susceptibility testing.

Antimicrobial susceptibility testing

The disk diffusion method on Mueller–Hinton agar was employed to evaluate susceptibility to 19 antimicrobial discs. The antibiotic discs used were purchased from HiMedia, India, contain the following: Gentamycin (G) (10 µg /disc), ciprofloxacin (Cf5) (5 µg /disc), levofloxacin (Le5) (5 µg /disc), cefuroxim (Cxm30) (30 µg/disc), pipracillin (Prl100) (100 µg/disc), cotrimoxazole (Smx25) (25 µg/disc), cefotaxime (Ctx30) (30 µg/disc), tazobactams (Tpz110) (110 µg/disc), colistin (Ct10) (10 µg/disc), ampicillin (Am10) (10 µg/disc), tetracyclin (Te30) (30 µg/disc), cefoxitin (Fox30) (30 µg/disc), tobramycin (Tb10) (10 µg/disc), cefazolin (Cz30) (30 µg/disc), cefaclore (Cec30) (30 µg/disc), ceftazidime (Ca30) (30 µg/disc), moxycillin (Am30) (30 µg/disc), ofloxacin (Of5) (5 µg/disc) and imipenem (Ipm10) (10 µg/disc). Zones of inhibition were read and compared with the values of susceptibility interpretive breakpoints issued by the National Committee for Clinical Laboratory Standards (NCCLS, 2013) to determine the degree of susceptibility to each antibiotic tested on each isolate test [31].

Molecular manipulation

DNA preparation

A twelve DNA extract of *A. baumannii* isolates were prepared with small scale chromosome extraction using Wizard Genomic DNA purification kit (Promega, USA) in accordance with the manufacturer's instructions.

Amplification of ITS

The bacterium-specific universal primers 1512F5`GTCGTAACAAGGTAGCCGTA3` and 6R 5`GGGTTYCCCCRTTCRGAAAT3` where Y is C or T and R is A or G [32] were used to amplify a DNA fragment using Gene Amp PCR system 9700 (Applied Biosystems). The DNA fragment encompassed a small fragment of the 16S rRNA gene region, the ITS, and a small fragment of the 23S rRNA gene region. The 5′ end of primer 1512F is located at position 1493 of the 16S rRNA gene, and the 5′ end of primer 6R is located at position 108 downstream of the 5′ end of the 23S rRNA gene (*Escherichia coli* numbering).

Using PCR reaction mixtures of a total volume of 50µl were prepared for all samples as follow: 5µl of each primer, 2.5 µL of DNA template, 25 µL of Go Taq Green Master Mix (Promega, USA), and 2 X, 12.5 µl of Nuclease-free water (up to 50 µl total volume). PCR was carried out in a with an initial denaturation step at 94°C for 2 minutes followed by 35 cycles of denaturation (94°C for 1 minute), annealing (62°C for 1 minute) and extension (72°C for 1 minute). The reaction was terminated with an extended elongation step (72 °C for 7 minutes). PCR products were purified using a Wizard SV gel and PCR clean-up System (Promega, USA). The amplified fragments obtained were electrophoretically separated in 2% agarose gels.

Identification of *A. baumannii* isolates by ITS sequencing

DNA sequencing of the ITS of the twelve clinical isolates that were identified as *A. baumannii* with the API 20 NE system (bioMérieuxVitek) was car-

ried out at the automated DNA sequencing facility using the TaqDyeDeoxiTerminator Cycle Sequencing Kit, and the sequence was analyzed in an automatic DNA sequencer in Korea (373A; Applied Biosystems). During analysis of the sequences obtained, the portions of the 16S and 23S rRNA gene regions were removed from the sequence data to obtain the exact ITS sequences. ITS DNA sequences of the isolates along with sequences of known *Acinetobacter* geno-species prototype strains retrieved from the GeneBank were aligned using molecular evolutionary genetic analysis programs (Mega 5.10).

Identification of the isolates by ARDRA

The ARDRA method was carried out as described previously [18, 33]. Briefly, the amplified 16S rRNA gene was obtained by Gene Amp PCR system 9700 (Applied Biosystems). The sequences of the primers were 59TGGCTCAGATTGAAC GCTGGCGGC39 (59 end of the 16S rRNA gene) and 59TACCTTGT-TACGA CTTCACCCCA39 (39 end of the 16S rRNA gene). Amplification was performed under the following conditions: initial denaturation step for 6 min at 94°C followed by 35 cycles of denaturation (94°C for 45 s), annealing (60°C for 45 s), and extension (72°C for 1 min).The reaction was terminated with an extended elongation step (72°C for 7 min). Separate aliquots were digested with four restriction endonucleases *Cfol, AluI, RsaI and MspI* (Promega, USA). The fragments obtained by digestion with each enzyme were electrophoretically separated in 2.5% agarose gels. Species identification was done by comparing the profiles consisting of the combination of restriction patterns generated with the different enzymes to those of a library of profiles of strains of described named and unnamed species [18,33] (http://allserv.rug.ac.be/~mvaneech/ARDRA/Acinetobacter.html).

Results

Bacterial isolates

The biocharacteristics of the 12 clinical isolates are shown in **Table 1**. Re-identification of the 12 clinical isolates using presumptive method for phenotypic characterization of *Acinetobacter spp* by API 20 NE system showed that all isolates belonged to *A. baumannii* with confidence levels >90%.

Antibiotic susceptibility testing

A. baumannii isolates revealed 100% resistance to the following investigated antibiotics (Gentamycin, cefuroxim, cotrimoxazole, cefotaxime, tazobactams, ampicillin, tetracyclin, cefoxitin, cefaclore, ceftazidime, amoxycillin, cefazolin and pipracillin, cirofloxacin, tobramycin, oflacacin, and levofloxacin, while all these were susceptible to colistin and imipenem.

Amplification of ITS fragments

The ITS fragments of 12 *A. baumannii* (genomic) species were amplified by PCR with primers 1512F and 6R. A single amplification product indicated a single amplicon of very constant length was observed for all isolates, and all the ITS fragments were 607bp which referred to *A. baumannii* (**Figure 1**). These results confirm the close similarity between all 12 strains obtained from two different hospitals.

Identification of clinical *A. baumannii* by ITS DNA gene sequencing

The twelve sequencing analysis revealed that the 5′ end of the ITS sequences were ACGAAAGATT, whereas the 3′ end sequence were GGGGTTGTAT which confirmed the identification of *A. baumannii*. The BLAST program in National Center for Biotechnology Information was used to align the 16S-23S rRNA intergenic spacer sequence of the new isolates with previously published sequences in the public database. ITS sequence analysis showed that

Table1. Phenotypic Characteristics and Antibiogram of the twelve clinical isolates of *Acinetobacter baumannii*

Patients' Features	Sample No.											
	1	2	3	4	5	6	7	8	9	10	11	12
Gender/age	F/51	F/70	M/62	M/42	M/47	F/35	F/35	F/78	F/14	F/< 1	M/58	M/34
Sample source	wound	wound	sputum	sputum	wound	wound	wound	blood	wound	CSF	wound	blood
Phenotypic Characteristics												
Gram staining	Gram-negative coccobaccilli											
Growth at 37ºC, 42ºC, 44 ºC	Heavy growth after 24 hour of incubation at 37ºC, 42º C and 44 ºC											
Catalase	All isolates were catalase +											
Oxidase	All isolates were oxidase -											
TSI	Alkaline/ Alkaline											
Glucose oxidation*	All isolates were Glucose oxidizers											
Gelatin hydrolysis	All isolates liquefied gelatin											
API 20NE	"Excellent" identification for *Acinetobacter baumannii* with 94.8%.											
Antibiogram												
R	Gentamycin, Cefuroxim, Cotrimoxazole, Cefotaxime, Tazobactams, Ampicillin, Tetracyclin, Cefoxitin, Cefaclore, Ceftazidime, Amoxycillin, Cefazolin, Pipracillin, Cirofloxacin, Tobramycin, Oflacacin, Levofloxacin											
S	Colistin , Imipenem											

*Acinetobacter isolates were tested for their abilities to oxidize glucose in Hugh and Leifson's medium

there was a strong similarity (86-88%) between the isolates and representative strains of the genus *Acinetobacter* in gene bank using the blast tool.

## Identification of clinical isolates by ARDRA

The 16S rRNA gene (16S rDNA) was enzymatically amplified for all the 12 isolates belonging to the *A. baumannii*, and the amplicon was restricted independently with the enzymes *CfoI, AluI, RsaI* and *MspI*. Restriction patterns were analyzed by 2.5% agarose gel electrophoresis which represents digestion pattern for 3 isolates, giving an overview of all of the patterns observed and all 12 isolates showed identical patterns for all restriction enzymes' digest (**Figure 2**). The pattern interpretation deduced that the 12 isolates are *Acinetobacter baumannii* using band analysis library at (http://allserv.rug.ac.be/mvaneech/ARDRA/Acinetobacter.html).
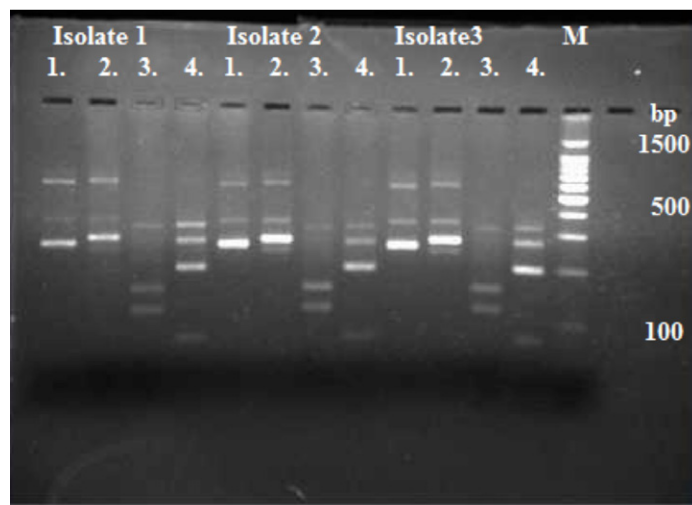
Discussion

This study describes and compares the molecular relationships and antibiograms of *A. baumannii*
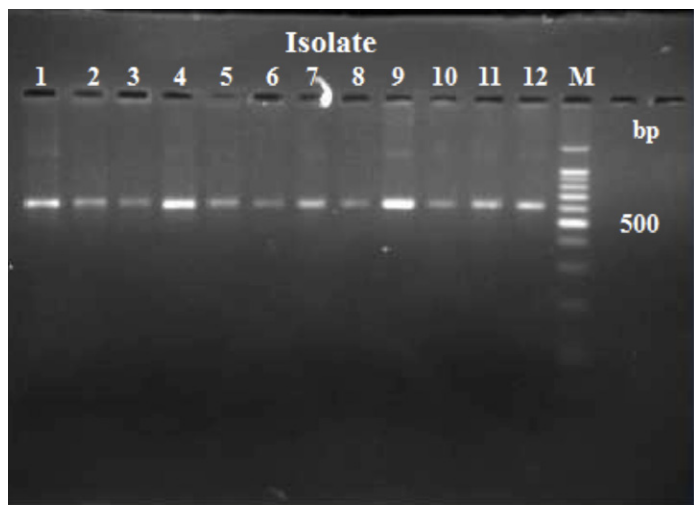
Figure 1. Amplification of *Acinetobacter baumannii* with primers 1512F and 6R and separation of the PCR products by 2% agarose gel electrophoresis. Lanes: M, 100-bp DNA ladder.

Figure 2. Restriction patterns of the amplification products obtained from 3 isolates amplicon of *Acinetobacter baumannii*. A total of 8 ml of the postamplification reaction mixture was digested with 1. *RsaI*, 2. *CfoI*, 3. *AluI*, 4. *MspI* and run in a 2.5% agarose gel. Lane designations indicate restriction, patterns. Lane M, 100-bpair ladder molecular weight marker.

isolates from patients hospitalized in two intensive care units of 2 major hospitals in Amman, Jordan. The phenotypic characterization results indicated that all isolates were belonging to *A. baumannii*. This preliminary result showed apparently that these *A. baumannii* isolates might be belonged to same clone. While genus-level characterization is usually sufficient for clinical diagnostic purposes, but it is not enough to prove the common source of isolates or their true genomic relationship. In addition, phenotypic methods are not enough to track transmission of isolates in hospital or help to understand the mechanisms of *Acinetobacter* infections. The original sources of the infecting organisms, their clone similarity, and geographical spread are considered an important requirements for the development of appropriate infection control measures.

Therefore, 16S-23S rRNA gene intergenic spacer (ITS) region as a genotypic method was performed for the genomic identification of these isolates. The results of this study demonstrated a high degree of conservation of both the ITS length and the ITS se-

quence of the twelve isolates which are a common feature in all species of *Acinetobacter spp* [12]. Restriction analysis of the amplified 16S rRNA gene (16S rDNA) was performed for more specific differentiation. It was observed that multiple ARDRA profiles may occur among different strains of the same species [12]. However, the conserved restriction enzyme analysis revealed that the 12 clinical isolates belonged to the same genomic group of *Acinetobacter baumannii* complex.

The application of ARDRA for the identification of *Acinetobacter* species has several advantages over phenotypic identification. ARDRA considered rapid and reliable and universally applicable method for identification of most of the *Acinetobacter* genomic species, thus contribute to better understanding of the clinical importance and epidemiology of this organism [17]. Summation of all phenotypic, antibiogram, and genotyping results suggested that there might be a common origin of infection in both JUH and KHCC. This study demonstrated the same genotype causing infection /colonization at the two major hospitals in Amman. The potential

sources of infection might be a common environmental reservoir that spread the organism such as water supply, ventilator, hospital staff, intravenous lines or monitoring devices, surgical drains or indwelling urinary catheters. To investigate the potential source of *Acinetobacter* nosocomial infection in hospital, samples should be taken from all those possible sources and patients for investing the genotypes of isolates by other advance genetic methods. An important drawback of this study is a lack of any tracing information for the movement of patients within and outside the hospital and no tracing information on visitors to the infected patients. However, a recent study carried at the JUH , found that *A. baumannii* isolates from hospital environmental and patients' respiratory tract sources have a common biological characteristics including much similar multidrug resistance patterns [5].

In conclusion, this study suggests that all 12 *A. baumannii* isolates have similar phenotypic and genotypic characteristics, although *A. baumannii* complex might be phenotypically homogeneous but genotypically heterogeneous.

Acknowledgment

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