Molecular Methods for Identification of *Acinetobacter* Species by Partial Sequencing of the *rpoB* and 16S rRNA Genes

**ABSTRACT**

**Background:** *Acinetobacter* spp. is a diverse group of Gram-negative bacteria which are ubiquitous in soil and water, and an important cause of nosocomial infections. The purpose of this study was to identify a collection of *Acinetobacter* spp. clinical isolates accurately and to investigate their antibiotic susceptibility patterns.

**Materials and Methods:** A total of 197 non-duplicate clinical isolates of *Acinetobacter* spp. isolates identified using conventional biochemical tests. The molecular technique of PCR-RFLP and sequence analysis of *rpoB* and 16S rRNA genes was applied for species identification. Antimicrobial susceptibility test was performed with a disk diffusion assay.

**Results:** Based on 16S rRNA and *rpoB* genes analysis separately, most of clinical isolates can be identified with high bootstrap values. However, the identity of the isolate 555T was uncertain due to high similarity of *A. grimontii* and *A. junii.* Identification by concatenation of 16S rRNA and *rpoB* confirmed the identity of clinical isolates of *Acinetobacter* to species level confidently. Accordingly, the isolate 555T assigned as *A. grimontii* due to 100% similarity to *A. grimontii.* Moreover, this isolate showed 98.64% to *A. junii.* Besides, the identity of the isolates 218T and 364T was confirmed as Genomic species 3 and *A. calcoaceticus* respectively. So, the majority of *Acinetobacter* spp. isolates, were identified as: *A. baumannii* (131 isolates, 66%), *A. calcoaceticus* (9 isolates, 4.5%), and *A. genomospor* 16 (8 isolates, 4%). The rest of identified species showed the lower frequencies. In susceptibility test, 105 isolates (53%), presented high antibiotic resistance of 90% to ceftriaxone, piperacillin, piperacillin tazobactam, amikacin, and 81% to ciprofloxacin.

**Conclusion:** Sequence analysis of the 16S rRNA and *rpoB* spacer simultaneously was able to do identification of *Acinetobacter* spp. to species level. *A. baumannii* was identified as the most prevalent species with high antibiotic resistance. Other species showed lower frequencies ranged from 4 to 9 strains.

**Keywords:** Nosocomial infections, PCR-RFLP, Phenotypic tests, Susceptibility testing

**INTRODUCTION**

*Acinetobacter* spp. is Gram-negative coccobacilli with wide distribution in environmental sources such as soil and water. They are also common organisms found in the hospital environment [1]. Recent molecular studies have shown 31 distinct species with valid names among the genus *Acinetobacter.* Besides, the genus comprises a number of taxa including species with published names [2,3]. Of these, *A. calcoaceticus,* *A. baumannii,* *A. pittii,* and *A. nosocomialis* (genomic species 1, 2, 3 and 13TU, respectively) are genetically and phenotypically very similar [4].

During the last 20 years, *Acinetobacter* species have emerged as opportunistic and important nosocomial pathogens that are associated with hospital acquired infections [5]. *A. baumannii* is the most important species is responsible for a significant proportion of nosocomial infections, including urinary tract infections, endocarditis, surgical-site infections, meningitis, septicemia, and ventilator-associated pneumonia among patients in intensive care units [6]. *A. baumannii* has more recently become a cause for major concern in clinical practice due to its high level of antimicrobial resistance. In particular, the worldwide emergence of the resistance to carbapenems [7], which have been accounted as the most effective antimicrobial agents for the treatment of infections caused by multidrug resistant (MDR), is reported increasingly [8]. Outbreaks of carbapenem-resistant *A. baumannii* were recently reported from Pakistan, Korea and China [9-11]. Due to growing importance of *Acinetobacter* species in hospital infections and particularly those with multidrug resistance capacity, the precise identification of the species is important to elucidate the ecology, epidemiology, and pathology of these species. Conventional phenotypic tests are proved to be unable to identify the *Acinetobacter* isolates to the species level [4]. However, in recent years, species identification has been made possible by using the developed molecular techniques [12]. These methods include the currently identification methods based on 16S rRNA and RNA polymerase B subunit (*rpoB*) genes sequences, for the description of *Acinetobacter* species. Both methods have been reported to be useful for molecular characterization of bacteria including *Acinetobacters* [13,14].

The objectives of the present study were to apply the molecular methods for the species identification of the *Acinetobacter* spp. clinical isolates and to determine their antibiotic susceptibility patterns.

**MATERIALS AND METHODS**

**Bacterial isolates**

A total of 197 non-duplicate isolates from a wide range of clinical samples were collected from Golestan and Imam Khomeini teaching Hospitals in Ahvaz and Sina Hospital in Tehran from November 2011 to January 2013. Clinical sources of the isolates were blood, endotracheal, urine, wound, sputum, exudates, percutaneous endoscopic gastrostomy (PEG), pleural fluid, cerebrospinal fluid (CSF), and soft tissue, though the majority of strains were isolated from endotracheal tube and blood. The isolates were kept in Trypticase Soy Broth (Merck, Germany) containing 20% glycerol at -80°C until use. Conventional phenotypic tests including growth on MacConkey agar (Merck, Germany), sugar fermentation, motility, catalase, oxidase, and other standard recommended tests were used to identify the genus of the isolates [15].
Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by agar disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [16]. The following antimicrobial agents were tested: ciprofloxacin (30µg), piperacillin (100µg), gentamycin (10µg), amikacin (30µg), trimethoprim sulfamethoxazole (25µg), meropenem (10µg), piperacillin tazobactam (100/10µg), ceftazidime (30µg), ceftixime (30µg), polymyxinB (20µg) and tetracycline (30µg) [MAST Co., UK]. A. baumannii ATCC 16906 was used as control strain. Determination of MDR and extensively drug resistance (XDR) was based on the CLSI criteria reported elsewhere [17].

Molecular identification

DNA extraction

All the isolates were grown on Muller Hinton Agar (Merck, Germany) and were incubated for 48 hours at 37°C under aerobic conditions and the bacterial colonies were then used for DNA extraction. The boiling method described by Higgins et al., [18], was used to extract the DNA from the bacterial colonies.

Fingerprinting of the 16S rRNA gene by PCR-RFLP analysis for strain clustering

Amplification of the 16S rRNA gene (1500 bp) was performed by using universal primers 27F (5’-AGAGTTTGATYMTGGCTCAG-3’) and 1525R (5’-AAGAGGAAGGATCAGCC-3’). PCR protocol was described elsewhere [14,19]. The PCR products were electrophoresed on a 2% agarose gel containing 0.5 µg/ml ethidium bromide (Qiagen, USA) to determine the size of the product. Both negative and reagent controls were included in each PCR run. The product was photographed by using the gel documentation system (UV Tech, UK). All PCR products were then digested by Hae III and Tag I restriction enzymes (Fermentas, Canada). Enzymatic digestion of the PCR products was performed according to the manufacturer’s instructions. In brief, 1 µl of the amplicon was treated separately with 2 units of Tag I or Hae III enzymes plus 10 µl corresponding buffer and were overnight incubated at 37°C. The digested reactions were then placed at 65°C for 30 minutes to avoid further enzymatic digestion. Ten microliters of digestion products were separated by electrophoresis on a 3% agarose gel containing 0.5 µg/ml ethidium bromide. A. baumannii ATCC16906 was used as a positive control. Restriction patterns were analysed visually comparing with the identity of the isolate 55ST was uncertain due to high similarity of A. grimontii. Similarly on the basis of 16S rRNA gene, the isolates 218T and 364T were unidentifiable due to identical 16S rRNA sequence of genomic species (Gen.sp.) 3 and A. calcoaceticus.

Sequence analysis of zone 1 of the rpoB gene for representative isolates from each cluster in RFLP grouping, identified most of the isolates to species level with high similarity. Based on rpoB gene, clinical isolate 55ST showed 100% and 99% similarity to those of A. grimontii, and Gen. sp. 5 (A. junii) respectively. Based on rpoB gene, the isolates 218T and 364T were identifiable to species level with high confidence of Gen. sp.3 A. calcoaceticus.

Identification by concatenation of 16S rRNA and rpoB confirmed the identity of clinical isolates of Acinetobacter to species level. Accordingly, the isolate 55ST assigned as A. grimontii due to 100% similarity to A. grimontii [Table/Fig-2,2]. Moreover, this isolate showed 98.64% (24 nucleotide mismatches) to A. junii. On the other hand, the identity of the isolates 218T and 364T was confirmed as Gen. sp. 3 and A. calcoaceticus respectively [Table/Fig-4].

DISCUSSION

Acinetobacter spp. and mainly A. baumannii are associated with hospital outbreaks worldwide. These organisms are particularly problematic due to the large number of MDR strains that have become endemic in hospital settings which is a growing concern in many countries [21,22].

In the present study, A. baumannii was the most prevalent (66.5%) Acinetobacter species. Several studies have investigated the distribution of Acinetobacters in clinical specimens at the species level, and considerable differences in outcome have been reported. In a recent report by Lee et al., [23], about 80% of their Acinetobacter isolates were identified as A. baumannii, which was higher than our findings. This rate was much lower in the study of Karah et al., [12], as 24% and in the study of Boo et al., [24], as 22%. In the latter study, A. pittii was the most frequent species found, representing 39% of their 114 isolates, whereas only 4 isolates in our study were identified as A. pittii, which the difference is considerable. This inconsistency may be explained either by the use of different interval surveillance cultures in their study or by the use of different methods for species identification. However, whether these differences are due to geographic, methodological, or epidemiological differences between the studies is hard to say.

Our finding represented extremely high rate of MDR as 81% and lower rate of XDR as 13%. This was in line with a report by Joshi et al., with more than 75% MDR among their Acinetobacter isolates.

The GenBank accession numbers for the sequences of the 16S rRNA and rpoB genes of the representative isolates are KM281495-KM281506 and KM668180-KM668191 respectively.
Patients are infected with the same strain, which is in line with the practice regarding antimicrobial therapy in isolates in latter studies is probably the restricted use of antibiotics, lower rate of MDR as 5.6% and 7.7% respectively [26,27]. A likely [25]. Similarly a recent report from Pakistan represented higher strain [9]. Although there are some discordant reports with much lower rate of MDR as 5.6% and 7.7% respectively [26,27]. A likely explanation for the relatively low level of resistance of Acinetobacter isolates in latter studies is probably the restricted use of antibiotics, which is in line with the practice regarding antimicrobial therapy in their countries, the strict isolation of patients with MDR isolates, and the immediate beginning of infection control measures when several patients are infected with the same strain.

### Table/Fig-1: Antibiotic susceptibility patterns of clinical isolates of Acinetobacter spp:

<table>
<thead>
<tr>
<th>Isolates</th>
<th>AMK</th>
<th>CIP</th>
<th>CTZ</th>
<th>PMB</th>
<th>MRP</th>
<th>TMP-SMX</th>
<th>CTX</th>
<th>TAZ</th>
<th>PIP</th>
<th>GEN</th>
<th>TCN</th>
<th>MDR Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.parvus</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>A.johnsonii</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>XDR</td>
</tr>
<tr>
<td>A.calcoaceticus</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>MDR</td>
</tr>
<tr>
<td>A.gen.sp. 16</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
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<td>R</td>
<td>R</td>
<td>R</td>
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<td>R</td>
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<td>MDR</td>
</tr>
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<td>A.gen.sp. 9</td>
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<td>R</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>MDR</td>
</tr>
</tbody>
</table>

### Table/Fig-2: Restriction patterns and clustering of clinical isolates of Acinetobacter spp. by 16S rRNA RFLP

<table>
<thead>
<tr>
<th>Number of isolates</th>
<th>Hae III</th>
<th>Tag I</th>
<th>RFLP type</th>
</tr>
</thead>
<tbody>
<tr>
<td>131</td>
<td>100/170/220/250/300/400</td>
<td>100/220/300/320/480</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>100/220/250/350/400</td>
<td>220/350/370/470</td>
<td>B</td>
</tr>
<tr>
<td>4</td>
<td>50/120/250/300/400</td>
<td>200/310/400/600</td>
<td>C</td>
</tr>
<tr>
<td>8</td>
<td>100/170/220/250/300/400</td>
<td>120/250/320/480/600</td>
<td>D</td>
</tr>
<tr>
<td>4</td>
<td>100/170/220/250/300/400</td>
<td>70/180/420/610</td>
<td>E</td>
</tr>
<tr>
<td>9</td>
<td>100/170/220/250/300/400</td>
<td>70/180/320/610</td>
<td>F</td>
</tr>
<tr>
<td>10</td>
<td>70/180/230/400</td>
<td>90/160/400/520</td>
<td>G</td>
</tr>
<tr>
<td>5</td>
<td>80/170/200/250/300/390</td>
<td>220/330/320/450</td>
<td>H</td>
</tr>
<tr>
<td>4</td>
<td>90/170/220/250/470</td>
<td>100/290/300/320/550</td>
<td>I</td>
</tr>
<tr>
<td>6</td>
<td>100/220/250/300/550</td>
<td>50/280/300/320/450</td>
<td>J</td>
</tr>
<tr>
<td>4</td>
<td>170/210/250/400/630</td>
<td>90/230/300/320/400</td>
<td>K</td>
</tr>
<tr>
<td>4</td>
<td>170/320/450/510</td>
<td>80/120/300/320/390</td>
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<tr>
<td>4</td>
<td>170/210/250/420/680</td>
<td>90/210/300/320/400</td>
<td>M</td>
</tr>
</tbody>
</table>

Identification to species level within the genus Acinetobacter is often problematic. Currently Acinetobacter species are defined on
The 16S rRNA and rpoB spacer simultaneously was able to do identification of Acinetobacter spp. to species level. A. baumannii was identified as the most prevalent species with high antibiotic resistance. Other species showed lower frequencies ranged from 4 to 9 strains.

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