Characterization of carbapenem-resistant Acinetobacter calcoaceticus–baumannii complex isolates from nosocomial bloodstream infections in southern Iran

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Acinetobacter baumannii is an important opportunistic bacterial pathogen responsible for serious infections in hospitalized patients. From a total of 78 consecutive non-repetitive Acinetobacter spp. isolates from patients with blood infections, 61 were carbapenem resistant, which were positive for blaOXA-51-like (96.7 %), blaOXA-23-like (77 %), blaOXA-58-like (8.1 %) and blaOXA-40-like genes (32.8 %) by multiplex PCR. The isolates were identified as A. baumannii (n=59) and Acinetobacter nosocomialis (n=2). Also, we found a case of Acinetobacter junii, causing bacteraemia, that possessed the IMP gene. High levels of resistance were observed to fluoroquinolones, aminoglycosides, tigecycline and to the beta-lactam antibiotics, including piperacillin/tazobactam and ampicillin/sulbactam. ISAba1 was present in 96.7 % of all Acinetobacter calcoaceticus–baumannii complex (Acb) isolates. Also, 33 (54.1 %) and 23 (37.7 %) isolates harboured ISAba1 upstream of blaOXA-23-like and blaOXA-51-like genes, respectively, though this was not observed in A. nosocomialis isolates. No relationship was observed between the presence of ISAba1 upstream of oxacillinase genes and the level of carbapenem resistance in all Acb isolates. Only two genes encoding metallo-beta-lactamase (VIM, SPM) were detected in all Acb isolates. This suggests that carbapenem resistance in blood-isolate Acb is mostly due to the presence of acquired carbapenemases. This is the first report from Iran on the identification of A. nosocomialis isolates that possess multiple oxacillinase genes and lack upstream ISAba1.

INTRODUCTION

Acinetobacter baumannii has emerged as a major pathogen of nosocomial infections in critically ill patients and is mostly the cause of septicemia, pneumonia and urinary tract infection following hospitalization of patients with more severe illnesses (Bergogne-Bérézin & Towner, 1996).

The genus Acinetobacter currently consists of 43 genomic species (http://www.bacterio.net/acinetobacter.html). The clinically relevant species include A. baumannii and two other species, Acinetobacter nosocomialis and Acinetobacter pittii (formerly known as Acinetobacter genomic species 3 and 13 sensu Tjernberg and Ursing, respectively). These three species are often grouped together alongside the environmental Acinetobacter calcoaceticus species and are, therefore, classified together into the so-called A. calcoaceticus–baumannii (Acb) complex (Nemec et al., 2011; Wisplinghoff et al., 2012). According to reported studies, apart from A. calcoaceticus, all members of this complex are found to be involved in nosocomial infections (Boo et al., 2009; van den Broek et al., 2009).

Identification of the species within the Acb complex is often difficult, especially in routine diagnostic laboratories, because they are genetically closely related and phenotypically very difficult to differentiate from each other (Gerner-Smidt et al., 1991). Therefore, up to 25 % of the isolates belonging to the Acb complex may have been misidentified as A. baumannii when using biochemical methods (Wisplinghoff et al., 2000). The RNA polymerase β-subunit (rpoB) gene sequences comprise one of the most useful tools for the identification of Acinetobacter spp., but it is unlikely that sequencing on this gene is to be used routinely. Other studies showed that multiplex PCR based on species-specific

Abbreviations: Acb, Acinetobacter calcoaceticus–baumannii complex; CRAB, carbapenem-resistant A. baumannii; IS, insertion sequence; MDR, multiple drug resistant; REP-PCR, repetitive extragenic palindromic PCR; XDR, extensively drug-resistant.
gyrB primers is a simple, specific and rapid method to reliably identify the species of the Acb complex (Higgins et al., 2010; Teixeira et al., 2013).

Of Acb complex species, A. baumannii is the most commonly found in clinical specimens and is resistant to multiple classes of antibiotics. Carbapenems are usually used to treat the infections caused by multiple drug resistant (MDR) A. baumannii. This class of antibiotics is not affected by most β-lactamases, such as Amber class A β-lactamase enzymes (extended-spectrum β-lactamas), which are capable of inactivating third-generation cephalosporins as well as monobactams (Navon-Venezia et al., 2005). For this reason, carbapenem resistance in A. baumannii strains has emerged as a major health concern worldwide. It has been shown that most of the carbapenem-resistant A. baumannii (CRAB) is due to the production of carbapenemases, especially those belonging to Amber class D β-lactamase enzymes (carbapenem-hydrolysing class D β-lactamases), which are mainly encoded by the blaOXA-51-like, blaOXA-23-like, blaOXA-40-like and blaOXA-58-like genes. Carbapenem resistance may occur due to the acquisition of an insertion sequence (IS), named ISAba1, whose presence upstream of OXA-type carbapenemase genes can provide promoter sequences that enhance expression of these genes (Poirel & Nordmann, 2006). In addition, class B metallo-beta-lactamases (VIM-, IMP- and SIM-types) have been sporadically reported in some parts of the world (Zarrilli et al., 2009). Other resistance mechanisms to carbapenems, including low permeability of the outer-membrane, target-site modifications (e.g. PBP alterations) and efflux-pumps, were also reported (Poirel & Nordmann, 2006).

The present study seeks to determine species distribution and drug susceptibilities among 61 carbapenem-resistant Acb complex isolates from patients with bloodstream infections admitted in a tertiary-care centre in Iran. It also investigates the most important β-lactamase-mediated mechanisms of carbapenem resistance among the isolates.

**METHODS**

**Bacterial isolates and species identification.** A total of 78 consecutive non-repetitive Acinetobacter spp. isolates were collected from patients with bloodstream infections admitted in Nemazee hospital, a large 800-bed tertiary-care hospital in the south of Iran from 2012 to 2013. Primary identification of A. baumannii isolates at the genus level was done by the biochemical methods and API 20NE system (bioMérieux). Species identification was performed using gyrB multiplex PCR, according to a previous study by Higgins et al. (2010). The identification was confirmed by partial rpOB gene sequence analysis (352 bp) (Gundi et al., 2009) on three non-A. baumannii isolates and on five strains of A. baumannii, randomly selected from the 59 A. baumannii strains, identified by multiplex PCR tests. Nucleotide sequence homology search was performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

A total of 61 Acb complex isolates, based on the results of disc diffusion and MIC tests, were selected for investigation of β-lactamase-mediated mechanisms of carbapenem resistance.

**Antimicrobial susceptibility testing.** Primarily, the disc diffusion method was used to assess susceptibility to 15 antimicrobial agents: levofloxacin, ciprofloxacin, tobramycin, imipenem, gentamicin, amikacin, chloramphenicol, piperacillin/tazobactam, tigecycline, ceftazidime, ampicillin/sulbactam, sulfamethoxazole/trimethoprim, rifampicin, meropenem, ceftazidime and colistin (M AST diagnostic).

All isolates were tested in Professor Alborzi Clinical Microbiology Research Center, using cation-adjusted Mueller–Hinton agar (BBL Becton Dickinson Microbiology Systems). Results were interpreted according to CLSI guidelines (CLSI, 2014).

**DNA extraction.** The micro-organisms were grown on blood agar plates overnight at 37 °C to ensure colony purity. Three or four bacterial colonies were taken from the blood agar plates, suspended in 250 μl of sterile ultrapure water, boiled for 10 min and centrifuged for 5 min at 12 000 g. The purity of the DNA was evaluated by the ratio of the absorbance at 260 and 280 nm (A260/A280). The supernatants were transferred to other 1.5 ml plastic tubes and were preserved at −70 °C until PCR amplification.

**Amplification of the carbapenem resistance genes in Acb complex isolates.** Detection of the four groups of OXA-carbapenemase genes (blaOXA-23-like, blaOXA-40-like, blaOXA-51-like and blaOXA-58-like) was carried out using a multiplex assay (Woodford et al., 2006). All strains were assayed for the ISAba1 sequence by PCR with primers ISAba1F and ISAba1R, giving rise to a 549 bp fragment. Also, the presence of the ISAba1 element upstream of the blaOXA-23-like and blaOXA-51-like genes was investigated by PCR using the primer pairs ISAba1F/OXA-23-R and ISAba1F/OXA-51-R (Segal et al., 2005; Turton et al., 2006).

To investigate the presence of the most common metallo-beta-lactamases, multiplex PCR was performed with five primer pairs, specific for each family of acquired metallo-beta-lactamases, to amplify the IMP, VIM, SPM, GIM and SIM genes, as previously described (Ellington et al., 2007).

**Genotype diversity by repetitive extragenic palindromic PCR (REP-PCR).** Genetic relationship of all isolates was determined by REP-PCR. In the REP-PCR method, the primer pair of REP1 (5’-HIIGGGCGGICATACGCC-3’) and REP2 (5’-ACGCTTATCAGGCTTAC-3’) was used and amplification PCRs were performed as described (Martín-Lozano et al., 2002). The amplified products were separated via electrophoresis on 1.5 % agarose gels and compared by visual inspection.

**RESULTS**

**Identification of Acinetobacter spp. and pattern of antimicrobial resistance**

In this study, 61 Acb complex isolates (78.2 %) were resistant to carbapenems amongst the 78 Acinetobacter spp. blood
isolates collected from hospitalized patients. On the basis of the results of API 20NE, gyrB multiplex PCR and rpoB gene analysis on all 78 isolates, *A. baumannii* accounted for 73 (93.5 %), *A. nosocomialis* for four (5.2 %) and *A. junii* for one isolate. Meanwhile, sequence analysis revealed 100 %, 99.7 % and 98.5 % identity with the rpoB gene for *A. baumannii*, *A. junii* and *A. nosocomialis*, respectively.

Of the 61 Acb complex isolates, *A. baumannii* was the most prevalent species accounting for 59 (96.6 %) isolates and *A. nosocomialis* accounted for two (3.4 %). As revealed, 80.1 % (59/73) of *A. baumannii* isolates were carbapenem resistant, while resistance among *A. nosocomialis* isolates was 50 % (2/4). MIC distributions and antimicrobial susceptibilities of 59 CRAB strains are shown in Table 1. A high level of carbapenem resistance was observed in all isolates (MIC$_{90}$ > 256 mg l$^{-1}$). The most active compound against *A. baumannii* isolates was colistin (100 % susceptibility). High-level resistance to fluoroquinolones, aminoglycosides, tigecycline and to the beta-lactam antibiotics, including piperacillin/tazobactam and ampicillin/sulbactam, was observed. All 59 (100 %) of the CRAB strains were determined as multidrug-resistant and 42 (80.7 %) strains were XDR. In contrast, only one of the *A. nosocomialis* isolates was XDR.

The *A. junii* isolate MIC for imipenem was 4.0 µg ml$^{-1}$, indicating intermediate susceptibility, and to meropenem was 8.0 µg ml$^{-1}$, showing resistance. Also, this isolate was susceptible to levofloxacin (0.125 µg ml$^{-1}$), ciprofloxacin (0.4 µg ml$^{-1}$), piperacillin/tazobactam (3 µg ml$^{-1}$), ampicillin/sulbactam (2 µg ml$^{-1}$) and colistin (0.19 µg ml$^{-1}$) and resistant to rifampicin (>32 µg ml$^{-1}$), ceftazidime (128 µg ml$^{-1}$), cefepime (64 µg ml$^{-1}$), gentamicin (16 µg ml$^{-1}$), amikacin (24 µg ml$^{-1}$) and sulfamethoxazole/trimethoprim (>32 mg ml$^{-1}$).

### Detection of carbapenemase genes

Analysis of the occurrence of OXA-encoding genes in 59 CRAB and two *A. nosocomialis* isolates is shown in Table 2. The *bla*$_{OXA-51}$-like genes were detected in all of the 61 carbapenem-resistant Acb complex blood isolates, except *A. nosocomialis* isolates, followed by *bla*$_{OXA-23}$-like (77 %), *bla*$_{OXA-40}$-like (32.8 %) and five *bla*$_{OXA-58}$-like (8.1 %). The co-existence of two OXA-encoding genes was detected in 55 isolates (90 %), with *bla*$_{OXA-51}$-like and *bla*$_{OXA-23}$-like genes in 37 isolates (60.6 %) and *bla*$_{OXA-51}$-like and *bla*$_{OXA-40}$-like genes in 18 isolates (29.5 %). One *A. nosocomialis* isolate had *bla*$_{OXA-23}$-like and *bla*$_{OXA-40}$-like genes. Moreover, four (6.6 %) isolates concomitantly possessed three OXA genes, *bla*$_{OXA-51}$-like, *bla*$_{OXA-23}$-like and *bla*$_{OXA-40}$-like. Also, three isolates harboured concomitantly all of the four OXA-encoding genes.

As observed, ISAba1 was present in all 61 (96.7 %) carbapenem-resistant Acb complex isolates, except two strains of CRAB (Table 2). Also, 33 (54.1 %) isolates harbouring ISAba1 upstream of *bla*$_{OXA-23}$-like and 23 (37.7 %) *bla*$_{OXA-51}$-like genes belonged to CRAB isolates. Although the presence of ISAba1 upstream of OXA genes confers resistance to carbapenems (Turton et al., 2006), no association between the presence of ISAba1 upstream of these two genes and level of carbapenem resistance was observed (Table 1). As mentioned above, none of the carbapenem-resistant *A. nosocomialis* isolates possessed ISAba1 upstream of the *bla*$_{OXA-23}$-like and *bla*$_{OXA-51}$-like genes, though ISAba1 elements existed in both isolates.

### Table 1. MIC distribution and resistance pattern of 59 *A. baumannii* strains isolated from patients with bloodstream infection

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Range</th>
<th>MIC$_{50}$</th>
<th>MIC$_{90}$</th>
<th>% Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEV</td>
<td>0.125 to &gt;32</td>
<td>16</td>
<td>&gt;32</td>
<td>95</td>
</tr>
<tr>
<td>CIP</td>
<td>0.2 to &gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>95</td>
</tr>
<tr>
<td>IMI</td>
<td>6 to &gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>100</td>
</tr>
<tr>
<td>AK</td>
<td>1.5 to &gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>94.2</td>
</tr>
<tr>
<td>PTZ</td>
<td>2 to &gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>93.3</td>
</tr>
<tr>
<td>TGC</td>
<td>0.75 to 48</td>
<td>2</td>
<td>4</td>
<td>74.5</td>
</tr>
<tr>
<td>SAM</td>
<td>2 to &gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>74.5</td>
</tr>
<tr>
<td>RP</td>
<td>0.38 to &gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>96.6</td>
</tr>
<tr>
<td>MER</td>
<td>8 to &gt;32</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>96.6</td>
</tr>
<tr>
<td>CO</td>
<td>0.125 to 1.5</td>
<td>0.38</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Distribution of carbapenemase genes and ISAba1 in carbapenem-resistant *Acinetobacter calcoaceticus–baumannii* complex blood isolates

<table>
<thead>
<tr>
<th>Genomic species</th>
<th>Oxa51</th>
<th>Oxa23</th>
<th>Oxa40</th>
<th>Oxa58</th>
<th>VIM</th>
<th>SPM</th>
<th>ISAba1</th>
<th>ISAba1 ▶ Oxa51</th>
<th>ISAba1 ▶ Oxa23</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. baumannii</em> (N=59)</td>
<td>59</td>
<td>46</td>
<td>18</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>57</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td><em>A. nosocomialis</em> (N=2)</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total (N=61)</td>
<td>59</td>
<td>47</td>
<td>20</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>59</td>
<td>23</td>
<td>33</td>
</tr>
</tbody>
</table>
The genes encoding metallo-beta-lactamase activity were detected in Acb complex isolates; VIM was observed in one A. baumannii and SPM in A. nosocomialis. Also, PCR results for carbapenemas in A. junii only revealed the presence of the IMP gene.

REP-PCR was carried out on genomic DNA and all CRAB isolates were separated into only two genotypes. However, the A. nosocomialis isolates showed diverse band patterns.

**DISCUSSION**

A. baumannii is an important opportunistic bacterial pathogen responsible for serious infections in hospitalized patients. In this study, a high rate of carbapenem resistance was found (61 out of 78). As reports from the present study hospital show, there has been a dramatic increase in the resistance to carbapenems for A. baumannii isolates, from 23 % in 2007–2008 (Japoni et al., 2011) to 78.2 % in 2012 (the present study) and 85.3 % in 2013–2014 (unpublished data). A worldwide study between 2005 and 2009, from 140 hospitals in 32 countries, revealed the overall non-susceptibility rate to imipenem and meropenem to be 45.9 and 48.2 %, respectively (Mendes et al., 2010).

The isolates were identified as A. baumannii (n=59), A. nosocomialis (n=2) and A. junii (n=1). To our knowledge, this is the first report on A. nosocomialis and A. junii blood isolates from Iran, although they have been previously reported from China, South Korea, Brazil, Singapore and Australia (Boo et al., 2006; Kim et al., 2012; Lee et al., 2012, Peleg et al., 2006; Teixeira et al., 2013). As other studies have demonstrated, the clinical findings of the infections caused by the above three species and respective antimicrobial susceptibilities may differ greatly (Lim et al., 2007; Wisplinghoff et al., 2012). The present study showed that A. baumannii is associated with greater imipenem resistance than in A. nosocomialis (81.1 % vs 50 %, \( P=0.003 \)). These results indicate the importance of precise species identification in Acb complex isolates in each hospital.

Only two genes encoding metallo-beta-lactamase activity (VIM, SPM) were detected in all the studied Acb complex isolates. This lends support to the finding that carbapenem resistance in A. baumannii isolates is mostly oxacillinase-mediated.

The \( bla_{OXA-51-like} \) genes were detected in all of the 61 carbapenem-resistant Acb complex blood isolates, except A. nosocomialis isolates, followed by 47 \( bla_{OXA-23-like} \) (77 %), 20 \( bla_{OXA-40-like} \) (32.8 %) and five \( bla_{OXA-58-like} \) (8.2 %). Previous studies reported that insertion of ISAba1 upstream of the \( bla_{OXA-51-like} \) and \( bla_{OXA-23-like} \) genes may enable the promoter to enhance gene expression, potentially contributing to increased levels of resistance to carbapenems (Turton et al., 2006). The prevalence of ISAba1 upstream of \( bla_{OXA-51-like} \) (37.7 %) genes among CRAB isolates was less than that of \( bla_{OXA-23-like} \) genes (54.1 %) but in total, 75 % of the isolates had ISAba1 upstream of one of them. Also, the data showed that carbapenem-resistant A. nosocomialis isolates harbour oxacillinase genes, although ISAba1 was not found upstream of these genes. In previous studies, \( bla_{OXA-23-like} \), \( bla_{OXA-40-like} \) and \( bla_{OXA-58-like} \) genes have been described for carbapenem-resistant non-A. baumannii isolates from China, Colombia, France, Germany and the Irish Republic (Bonnin et al., 2014; Boo et al., 2006; Evans et al., 2010; Wang et al., 2007). The present study demonstrated for the first time, the coexistence of oxacillinase genes in all carbapenem-resistant A. nosocomialis isolates and the existence of IMP in an A. junii blood isolate, in Iran. This A. junii isolate was not sensitive to any of the beta-lactam antibiotics, including the carbapenems, as well as aminoglycosides and SXT-SMZ.

We observed that the presence of the oxacillinase genes was associated with resistance to carbapenem in CRAB isolates. No correlation was observed between the ISAba1 upstream oxacillinase gene and level of carbapenem resistance (Table 1). Meanwhile, the high level of carbapenem resistance may be the result of other mechanisms such as modification of penicillin-binding proteins, porins or efflux systems (Poirel & Nordmann, 2006; Zavascki et al., 2010).

This study reports a large outbreak of XDR A. baumannii in southern Iran (75 %). Despite being highly sensitive to colistin (100 %), A. baumannii isolates with high resistance to different classes of antibiotics, including fluoroquinolones, aminoglycosides, tigecycline, piperacillin/tazobactam, ampicillin/subactam and rifampicin, were detected. The high antibiotic resistance could be due to selection pressure from high antibiotics use. Also, high resistance to many antimicrobials has become a serious challenge in both the choice of empiric therapy and indication for an alternative therapy (Table 1).

In conclusion, carbapenem resistance among A. baumannii isolates is increasing dramatically. The \( bla_{OXA-51-like} \) and \( bla_{OXA-23-like} \) genes have become a predominant carbapenem-resistance determinant in A. baumannii clinical isolates in southern Iran. ISAba1 was located in the promoter region of the \( bla_{OXA-51-like} \) and \( bla_{OXA-23-like} \) genes in a considerable number of isolates, but no correlation was observed between its presence and carbapenem resistance level. This is the first report from Iran on the identification of A. nosocomialis isolates from blood infections that possess multiple oxacillinase genes and lack upstream ISAba1 elements.

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