Dissemination of carbapenem-non-susceptible Acinetobacter baumannii isolates collected from educational hospitals in Qazvin province of Iran

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\textbf{Author contributions} 
AP designed the study, and SB and SA performed the experimental stages of the study. SS collaborated in statistical analysis steps. ZH and SB prepared a first draft of the manuscript, which was reviewed and revised by AP and RS. All the authors confirmed the final manuscript.

\textbf{Competing interests} 
The authors declare no conflicts of interest.

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\textbf{Abbreviations} 
OXA, oxacillin-hydrolyzing; MBL, metallo-\β-lactamases; rep-PCR, repetitive extragenic palindromic-PCR; PCR, polymerase chain reaction; UTIs, urinary tract infections; CRAB, carbapenem-resistant A. baumannii; UTIs, urinary tract infections; MBLs, Metallo-\β-lactamases; EOTA, ethylenediaminetetraacetic acid; IMP, Imipenem Metallo-\β-lactamase VIM, Verona Imipenemase; NDM, New-Delhi Metallo-\β-lactamase; GIM, German imipenemase; SPM, São Paulo metallo-\β-lactamase; CLSI, clinical and laboratory standards institute; ICU, Intensive Care Unit.

\textbf{Citation} 

\textbf{Abstract} 

\textbf{Background:} Acinetobacter baumannii (A. baumannii) is known as an opportunistic pathogen related to health-care-associated infection that has a high antibiotic resistance potential, notably against carbapenems that are widely used to combat A. baumannii infections. This study aimed to detect oxacillin-hydrolyzing (OXA) carbapenemases and metallo-\β-lactamases (MBL) among carbapenem-resistant A. baumannii isolated strains and to determine their clonal relationship by repetitive extragenic palindromic PCR (rep-PCR).

\textbf{Methods:} In the present study, a total of 211 non-repetitive isolates of A. baumannii were collected from Qazvin educational hospitals (2016–2017). The disk diffusion method was used to investigate the antibiotic susceptibility of studied strains, followed by the detection of MBL and OXA-type genes using polymerase chain reaction (PCR) and sequencing methods. The rep-PCR method assessed the clonal relationship of carbapenem-non-susceptible A. baumannii isolates.

\textbf{Result:} The obtained results showed that 87.2% and 86.7% of isolates were non-susceptible to imipenem and meropenem. The \textit{bla}\textsubscript{OXA-24} (93.5%) was the most frequent gene, followed by the \textit{bla}\textsubscript{OXA-23} (4.34%), \textit{bla}\textsubscript{IMP-1} (1.63%), and \textit{bla}\textsubscript{OXA-58} (0.54%). Meanwhile, \textit{bla}\textsubscript{OXA-58} and \textit{bla}\textsubscript{OXA-143} genes were not found. 81.5% and 66.1% of isolates contained ISAba1 upstream of the \textit{bla}\textsubscript{OXA-23} and \textit{bla}\textsubscript{OXA-58} genes, respectively. Rep-PCR results revealed the carbapenem non-susceptible isolates belonged to three distinct clones: A 171 (81%), B 34 (16.1%), and C 6 (2.8%).

\textbf{Conclusions:} The results indicated a high prevalence of carbapenem-non-susceptible A. baumannii, with the emergence of the \textit{bla}\textsubscript{OXA-24} gene as the most common gene and the notable prevalence of MBL genes. These results revealed the need for appropriate therapeutic and infection control strategies and monitoring susceptibility patterns for controlling A. baumannii infections.

\textbf{Keywords:} Acinetobacter baumannii; Carbapenemase; Metallo-\β-lactamases; Repetitive extragenic palindromic (rep)-PCR

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**Background**

*Acinetobacter baumannii* (A. baumannii) is the most crucial pathogenic species in the *Acinetobacter* genus, which is associated with a variety of hospital-acquired infections, especially for those suffering from underlying and chronic disorders such as diabetes and chronic pulmonary disease [1, 2]. This opportunistic pathogen is associated with wound infections, bloodstream infections, urinary tract infections (UTIs), skin and soft tissue infections, endocarditis, and abdominal abscesses [3, 4]. Carbapenems are a group of β-lactam antibiotics with extended activity versus penicillins and cephalosporins. These antibiotics include imipenem, meropenem, doripenem, and ertapenem [5]. Carbapenems are massively used against *A. baumannii* infections, but carbapenem-resistant *A. baumannii* (CRAB) is a concerning pathogen in hospital-associated infections. Several mechanisms may cause resistance to carbapenems: 1) point mutations in target genes that reduce or remove drug affinity; 2) efflux transport systems belonging to outer membrane proteins; 3) reduced autolysin activity; and 4) enzymatic activity named as β-lactamases [6]. Carbapenem-hydrolyzing β-lactamases of Ambler’s classifications B and D have emerged over the last decades. Metallo-β-lactamases (MBLs) belong to the class B carbapenemases, which were reported for the first time in Japan in 1988. Plasmids encode these enzymes and can be transferred among different species of bacteria, [7] and also have a metallic ion in their active site that is inhabited by ethylenediaminetetracetic acid (EDTA) and THOL components and includes: IMP (Imipenem Metallo-β-lactamase), VIM (Verona Imipenemase), NDM (New-Delhi Metallo-β-lactamase), GIM (German imipenemase), and SPm (São Paulo metallo-β-lactamase) [8]. Class D β-lactamase, carbapenem hydrolysis Oxacillinase (CHDL), is found in *A. baumannii* with carbapenemase activities, but cannot hydrolyze broad-spectrum cephalosporins and aztreonam. These enzymes are classified in the category 2D, and five phylogenetic subgroups of Group D β-lactamase have been know to contain the *bla*oxa-51 like groups and four acquired enzymes, including OXA-23, OXA-24/40, OXA-58, and OXA-143 [9]. Based on the previous studies, *A. baumannii* has a high prevalence in hospital settings, can cause different life-threatening infections, and has an important role in the emergence of antibiotic resistance, especially against carbapenems. Therefore, the present study was to assess the dissemination of carbapenem resistance-associated genes (MBLs and OXAs) in clinical isolates of carbapenem non-susceptible *A. baumannii* collected from various hospitals of the Qazvin province of Iran, and to determine their relationship by rep-PCR.

**Materials and methods**

**Bacterial isolates**

Two hundred and eleven isolates of *A. baumannii* were obtained from various hospital departments in Qazvin from July 2015 to June 2017. Obtained specimens included trachea, urine, blood, and wounds. To identify isolates, first, the clinical specimens were cultured on two blood agar plates (Qlab, Canada) and MacConkey agar culture media (Qlab, Canada) and then incubated at 37 °C overnight. Next, microbiological and biochemical tests were performed to identify *A. baumannii* species. Then, *A. baumannii* strains were confirmed using PCR and the presence of the *bla*oxa-51. All isolates were kept at −70 °C for further investigation. The current study was ethically approved by the Qazvin University of Medical Sciences ethics committee (code IR.QUMS.REC.1396.775).

**Phenotypic assessment of carbapenem resistant**

The test was applied using the standard disk diffusion and Kirby-Bauer method following the clinical and laboratory standards institute (CLSI M100S-Ed28) guidelines using imipenem (10 μg) and meropenem (10 μg) disks (MAST CO, UK). *A. baumannii* ATCC (American Type Culture Collection) 19603 was used as a standard strain for quality control.

**Detection of OXAs and MBLs encoding genes by PCR**

The amplification of *bla*oxa-51 like, *bla*oxa-23, *bla*NDM, *bla*vim, and *bla*SPM genes was performed by specific primers using PCR (Table 1). Extracting of total DNA was performed using the kit (Bioneer Company, South Korea) based on the manufacturer’s instructions. PCR amplification was performed using a thermocycler machine (Applied Biosystems, USA). The primers and annealing temperatures are shown in Table 1. [15, 26, 27, 28] Agarose gel electrophoresis was performed to assess the PCR products and visualized by using a gel documentation system (UVtec, UK).

**Bacterial clonal distribution assessment using rep-PCR**

In the present study, the rep-PCR method was used to analyze the clonal distribution in hospital departments. Rep-PCR reactions were made from a mixture of distilled water, 10X PCR buffer, 2 Taq DNA polymerase, primer reverse, primer forward, MgCl2, 4dNTP Mix (10 mol), and DNA template. PCR amplification process. PCR amplification wasperformed using a thermocycler(Applied Biosystems,

**Table 1 Primers used for OXAs and MBLs encoding gene detection**

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primer sequence (5′→3′)</th>
<th>Product (bp)</th>
<th>Annealing temperature (°C)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXA-51-F</td>
<td>TAAAGTTGAGCACTGCTGTC</td>
<td>324</td>
<td>51</td>
<td>[15]</td>
</tr>
<tr>
<td>OXA-51-R</td>
<td>GATTGAGCACTGCTGCTG</td>
<td>501</td>
<td>53</td>
<td>[15]</td>
</tr>
<tr>
<td>OXA-23-F</td>
<td>GATTGAGCACTGCTGCTG</td>
<td>246</td>
<td>55</td>
<td>[15]</td>
</tr>
<tr>
<td>OXA-24-F</td>
<td>GATTGAGCACTGCTGCTG</td>
<td>599</td>
<td>54</td>
<td>[15]</td>
</tr>
<tr>
<td>OXA-143-F</td>
<td>TGGCATTTTCGCACTGCTTCT</td>
<td>149</td>
<td>59</td>
<td>[26]</td>
</tr>
<tr>
<td>OXA-143-R</td>
<td>TAATCTGGAGGGGGGCAACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISAhs1-F</td>
<td>CAAGAAAAAGGTTG</td>
<td>227</td>
<td>54</td>
<td>[27]</td>
</tr>
<tr>
<td>ISAhs1-R</td>
<td>TGGATTGGAGCACTGCTG</td>
<td>174</td>
<td>53</td>
<td>[27]</td>
</tr>
<tr>
<td>IMP-1-F</td>
<td>TTGTGTTGAAAGGTTACG</td>
<td>210</td>
<td>52</td>
<td>[28]</td>
</tr>
<tr>
<td>IMP-1-R</td>
<td>TTATATCTGCACTGCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIM-1-F</td>
<td>GGGTTGCTGGTGGCACTGCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIM-1-R</td>
<td>TTATGTGGGCGATGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDM-1-F</td>
<td>GGTTGCGCGATGGTTTTCT</td>
<td>279</td>
<td>54</td>
<td>[27]</td>
</tr>
<tr>
<td>NDM-1-R</td>
<td>GGAATGGGACAGATGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPM-F</td>
<td>AAAATCGGCTGGCAAGAAGG</td>
<td>540</td>
<td>49</td>
<td>[28]</td>
</tr>
<tr>
<td>SPM-R</td>
<td>ACATAATCGGCTGGAAGAGG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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USA) and 45 °C was used for primer annealing for 55 seconds. Amplification products were assessed by gel electrophoresis. Similar band patterns of rep-PCR (less than two bands difference) were identified for the same clones.

Data analysis
SPSS version 22.0 (Inc, Chicago, IL, USA) and descriptive statistics were used to analyze the raw data.

Results
Patients, isolates, and antimicrobial susceptibility testing
Of 211 non-repetitive A. baumannii isolates, 184 (87.2%) and 183 (86.7%) were non-susceptible to meropenem and imipenem, respectively. One hundred and thirty-one (62.1%) isolates were obtained from males and 80 (37.9%) from females. Isolates were collected from trachea 100 (47.3%), urine 42 (19.9%), blood 29 (13.7%), sputum 29 (13.7%), and wound 12 (5.68%) specimens. In addition, the isolates were collected from patients in the intensive care unit (ICU) (122, 57.8%), internal medicine 43 (20.4%), infectious diseases 29 (13.7%), neurosurgery 11 (5.2%), and surgery 6 (2.8%) wards.

The PCR amplification of OXAs and MBLs encoding genes
Of 184 carbapenem non-susceptible isolates, 184 (100%), 172 (93.5%), and 8 (4.34%) were positive for blaOXA-51, blaOXA-23, and blaOXA-24, respectively. In this study, 6 (3.26%) isolates shared blaOXA-23 genes and blaOXA-24 genes. Also, 4 (2.17%) isolates were positive for MBL-encoding genes, of which 3 (1.63%) carried blaIMP-1, and 1 (0.54%) isolate was positive for blaVIM-1, blaOXA-48, and blaOXA-58. blaOXA-51, and blaNDM-1 genes were not found in the studied strains. Moreover, as shown in Table 2, blaOXA-51 gene was found to co-exist with blaOXA-23, in 165 (78.2%) isolates, and with blaOXA-24, and blaOXA-58, in 7 (3.3%) isolates. Also, co-occurrence of blaOXA-51 with blaVIM-1 was found in 3 (1.4%) isolates, with blaOXA-23 in 1 (0.5%) isolate and with blaOXA-24 in 1 (0.5%) isolate. In addition, all 172 (81.5%) isolates, which contained the blaOXA-23 gene, had ISAba1 upstream of the genes. Of 180 isolates containing the blaOXA-51 gene, 109 (66.1%) contained the ISAba1 sequence upstream of the gene.

Prevalence of three different bacterial clones in studied strains
Analysis of Rep-PCR showed that all carbapenem resistant isolates belonged to 3 distinct genotypes: A 171 (81%), B 34 (16.1%), and C 6 (2.8%). Furthermore, all the MBL carrying isolates belong to group A. The isolates that carried both blaOXA-51 and blaOXA-23 were more prevalent and belonged to group A (136, 64.5%) isolates, followed by group B (25, 11.8%) isolates, and group C (4, 1.9%) isolates. Moreover, the co-existence of blaOXA-51 with blaOXA-24 + blaOXA-23 was shown in 7 (3.3%), of which 5 (2.4%) and 2 (0.9%) were related to A and B groups, respectively.

Discussion
Carbapenems are most commonly used as last-line therapy against multidrug resistant Gram-negative pathogens [10]. Recently, the increased morbidity and mortality of carbapenem-resistant A. baumannii in hospital departments, particularly among those admitted to the ICU, has caused serious concerns [11]. In our study, out of 211 non-repetitive isolates, 122 (57.8%) were collected from patients admitted to the ICU, and it was found that prolonged hospitalization, extensive use of antibiotics, underlying health conditions, and the use of invasive devices such as mechanical ventilators and catheters promote A. baumannii infection.

In this study, 184 (87.2%) of A. baumannii isolates were non-susceptible to carbapenems, among those 184 (87.2%), 183 (86.7%) isolates showed resistant patterns against imipenem and meropenem, respectively. These reports are similar to the study conducted by Alaei et al. in Iran, which reported that 86% of clinical isolates of A. baumannii were resistant to carbapenem drugs [12]. In another study by Mohajeri et al., which was performed in Iran in 2013, resistance rates against imipenem (79.8%) and meropenem (75%) in the clinical isolates of A. baumannii were investigated [13]. Also, Shoja et al. in a study from Iran, revealed that 78.2% and 73.4% of A. baumannii isolates were non-susceptible to imipenem and meropenem, respectively [14]. According to those findings, there is a high rate of resistance to carbapenem drugs, and it can be attributed to the consequence of inappropriate and extensive use of carbapenem drugs to treat infections. This study revealed the importance of antimicrobial resistance surveillance systems for monitoring resistance patterns at both local and national levels.

According to the current study, blaOXA-51 (100%), blaOXA-23 (93.5%), and blaOXA-24 (4.34%) were the most prevalent genes. Azizi et al. showed that blaOXA-23 and blaOXA-58 genes were the most common in their A. baumannii isolates, and the isolates were negative for blaOXA-58-like genes [15]. In another publication documented from Tehran, Iran, Karmostaji et al. showed that 93.9%, 87.7%, 13.8%, and 1.6% of A. baumannii isolates harbored the blaOXA-51, blaOXA-23, blaOXA-24, and blaOXA-58 genes, respectively and also 92.3% and 96.2% of strains contained ISAba1 upstream of the blaOXA-51 and blaOXA-23 genes [16]. Also, Azimabadi et al reported that 87.5% and 9.7% of A. baumannii isolates were positive for blaOXA-51-like and blaOXA-23-like genes [17]. Altogether, these findings revealed the possible role of blaOXA-51 and blaOXA-23 genes in developing the carbapenems resistant strains in Iran. Carvalho et al. in a study which was conducted in Brazil, reported that blaOXA-23-like gene presence in 87.3% of A. baumannii isolates, and no isolates were blaOXA-23-Like, blaOXA-51-like, and blaNDM-1 positive [18].

Table 2 Clonal spread of OXAs and MBLs genes among clinical isolates of A. baumannii

<table>
<thead>
<tr>
<th>Resistance Genes</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXA-51 + OXA-23</td>
<td>136 (64.5)</td>
<td>25 (11.8)</td>
<td>4 (1.9)</td>
<td>165 (78.2)</td>
</tr>
<tr>
<td>OXA-51</td>
<td>25 (11.8)</td>
<td>7 (3.3)</td>
<td>2 (0.9)</td>
<td>34 (16.1)</td>
</tr>
<tr>
<td>OXA-51 + OXA-24 + OXA-23</td>
<td>5 (2.4)</td>
<td>2 (0.9)</td>
<td>-</td>
<td>7 (3.3)</td>
</tr>
<tr>
<td>OXA-51 + IMP-1</td>
<td>3 (1.4)</td>
<td>-</td>
<td>-</td>
<td>3 (1.4)</td>
</tr>
<tr>
<td>OXA-51 + OXA-24</td>
<td>1 (0.5)</td>
<td>-</td>
<td>-</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>OXA-51 + VIM-1</td>
<td>1 (0.5)</td>
<td>-</td>
<td>-</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>171 (81.1)</td>
<td>34 (16.1)</td>
<td>6 (2.8)</td>
<td>211 (100)</td>
</tr>
</tbody>
</table>

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Also, a study performed in Egypt by El Bannah et al. reported the presence of blaOXA-23 (100%) and blaOXA-22 (76.7%) genes among carbapenem resistant A. baumannii isolates [19]. Our findings also showed the co-occurrence of OXA-encoding genes as well as OXA and MBL-encoding genes, such as blaOXA-22, blaOXA-23, blaOXA-42, and blaOXA-51. Similar to our findings, Raj Joshi et al. showed the co-existence of blaOXA-22, blaOXA-23, and blaOXA-42 [20]. Consistent with the results of the current study, Huang et al. reported that 49 (74.6%) isolates carried both blaOXA-23 and blaOXA-22 [21].

In the current study, 3 (1.6%) and 1 (0.5%) isolates were positive for blaIMP-1 and blaOXA-23, respectively. However, we couldn’t detect blaOXA-42 and blaVIM-1 among targeted isolates. Similar to the findings of this study, Amin et al. in a study performed in Iran, reported that among clinical Acinetobacter isolates, 9.5%, 19%, and 5.4% were positive for blaOXA-22, blaOXA-23, and blaOXA-51 genes, respectively [22]. Also, Pourabbas et al. reported that 1.69% of clinical isolates of A. baumannii collected in Iran carried the blaOXA-23 gene [23]. Interestingly, Vijayakumar et al. showed that 34.24% of A. baumannii isolates collected in South India were positive for the blaOXA-23 gene, which suggests the high prevalence of MBL-encoding genes in India compared to our region [24]. In addition, Markelz et al. failed to detect MBL-encoding genes among clinical isolates of A. baumannii, which were collected from the USA [25]. Overall, it can be figured out that MBL-encoding genes are not prevalent among A. baumannii isolates compared to the OXA-encoding genes. Based on the rep-PCR obtained results from the current study, all carbapenem resistant A. baumannii isolates have shown 3 different genetic patterns which showed their related clonal distributions. Our rep-PCR findings showed that all studied strains belonged to three distinct clones, showing the clonal distribution of these resistant strains among studied hospitals. With a frequency of 171 (81%), clone A was the most prevalent, which suggests the clonal dissemination of the hospital-acquired infections and possible exchange of resistant elements in more than 80% of studied strains. Among all studied genes, the co-existence of blaOXA-23 and blaOXA-22 was the most prevalent, followed by the distribution of blaOXA-23 and clone A co-harborized with the blaOXA-23 and blaOXA-22.

Conclusion

According to our findings in studied A. baumannii isolate, carbapenem resistance due to the presence of OXA-type genes were high prevalent. Besides, the results revealed that blaOXA-23 and blaOXA-22 are the most prevalent genes, and MBL-type β-lactamases had low prevalence. Our study also indicated the clonal distribution of these resistant isolates in investigated hospital departments. In addition, using antibiotics collaborating with labs and concerning the carbapenem resistance in hospital-acquired strains is recommended. Moreover, our study revealed the emergence of possible carbapenem gene exchange in hospital-associated strains.

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