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Molecular Detection of Carbapenem Resistance in *Acinetobacter Baumannii* Isolated From Patients in Khorramabad City, Iran

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**Abstract:** Background: *Acinetobacter baumannii* is an opportunistic pathogen, which causes a wide range of infections in hospitals, especially in intensive care units. Nowadays, due to the high resistance of *Acinetobacter baumannii* to antibiotics, this study, in addition to the phenotypic and genotypic investigations of drug resistance, focused on determining the molecular types of *Acinetobacter baumannii* isolated from patients in Khorramabad city by the pulsed-field gel electrophoresis (PFGE) method.

**Materials and Methods:** In this cross-sectional study, 50 samples of *Acinetobacter baumannii* were collected from educational hospitals in Khorramabad city, Iran, from January to August 2015. They were identified in the laboratory using biochemical tests and culture methods. After determining the drug resistance pattern by the disc diffusion method and percentage of resistance genes to carbapenem, *Acinetobacter baumannii* isolates were analyzed using the PFGE method using the ApaI enzyme.

**Results:** The highest antibiotic resistance observed for *Acinetobacter baumannii* strains was against ampicillin-sulbactam (100%) and aztreonam (98%). The highest sensitivity was to polymyxin B (100%) and colistin (94%), and also to the OXA-51-like gene present in all samples. The OXA-23-like gene was positive in 44 (88%) samples. PFGE results showed that *Acinetobacter baumannii* strains had 33 different pulstype patterns, of which 27 patterns had more than one strain and 23 had only one strain.

**Conclusion:** Due to the high resistance of *Acinetobacter baumannii* and its ease of spread and its ability to transfer resistance genes, resistance control methods should be used in the disinfection of hospital areas. Hospital staff should observe hygiene standards and there should also be a reduction in antibiotic use.

**Keywords:** *Acinetobacter baumannii*, antibiotic resistance, pulsed-field gel electrophoresis (PFGE).

**1. INTRODUCTION**

*Acinetobacter baumannii* is a coccobacillus, gram-negative, negative oxidase, obligate aerobic, non-fermented, and non-motile [1]. With few requirements for growth, this bacterium can survive long-term in adverse, dry and aquatic environmental conditions [2]. *Acinetobacter baumannii* is widely distributed in the hospital environment and is easily transmitted among patients, especially patients admitted to the intensive care unit [3]. A wide range of infections, including urinary tract infections, meningitis, bacteremia, wound infections, skin and soft tissue infections, and respiratory infections are due to *Acinetobacter baumannii* activity [1, 4, 5].

Nowadays, *Acinetobacter baumannii* is resistant to most antibiotics, including aminoglycosides, carbapenem, cephalosporins and beta-lactams [6-8]. The factor that strengthens this resistance system is its intrinsic ability to survive long-term in all hospital settings, which has led to an increase of the prevalence of this bacterium in hospitals [9, 10].

One of the drugs used worldwide to treat this bacterium infection is the beta-lactam family of antibiotics [11, 12]. But as mentioned, these bacteria are resistant to beta-lactams, due to genetic elements. The most common genes associated with resistance to beta-lactam resistant isolates are...
ampC cephalosporinase genes, OXA type carbapenems, metallobeta lactamases, tracheal pumps, and introns [13-16]. Currently, carbapenems are the preferred antibiotics against multi-drug resistant *Acinetobacter* infections. However, resistance to carbapenems has been a major concern for public health, and there are limited therapeutic options to prevent the horizontal transfer of resistant genes and the inherent differential expression of the genes.

There are many methods for molecular typing of bacteria. Genotyping techniques are used in the *Acinetobacter baumannii* assimilation. Some of these methods are comprised of Pulsed Field Gel Electrophoresis (PFGE), Ribotyping, Random Amplification of Polymorphic DNA, Amplified fragment length polymorphism, and Repeatitive element sequence-based PCR(REP-PCR) [17]. In the meantime, the PFGE technique is considered as the gold standard method for determining the species of *Acinetobacter baumannii* [10, 18]. Although this method is complicated, its equipment is currently not only found in reference laboratories, but also in some of the advanced laboratories in hospitals. Typically, limited ApaI and Smal enzymes are used to cut chromosome DNA. Then, chromosomal portions created by electrophoresis are separated from each other, and fingerprinted graphs are compared either by eye or using computer programs [19].

Using this method, no strain of *Acinetobacter baumannii* had previously been isolated in Khorramabad. This study was conducted to analyze the phenotypic and genotypic resistance of this bacterium, with the aim of determining the molecular types of *Acinetobacter baumannii* isolated from patients in Khorramabad city by the PFGE method.

### 2. MATERIALS AND METHODS

#### 2.1. Bacterial Isolates

For this cross-sectional study, *Acinetobacter baumannii* isolates were collected from educational hospitals in Khorramabad, Iran, from January to August 2015. In the laboratory, after culture of the isolates on a MacConkey agar medium, *Acinetobacter baumannii* isolates were identified by standard biochemical tests.

#### 2.2. Antimicrobial Susceptibility Pattern

In order to investigate the resistance of *Acinetobacter baumannii*, disc diffusion tests were performed for 13 different antibiotics from MAST UK, including ceftotaxime(30 µg), colistin(10 µg), meropenem(10 µg), polymixin B(300 µg), aztreonam(30 µg), tetracycline(30 µg), cefepime(30 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), ampicillin-sulbactam(10/10 µg), amikacin (10 µg), tobramycin(10 µg), ceftriaxone (30 µg), and ciprofloxacin (5 µg), using the Antibiotic disc diffusion method. For the purpose of quality control of the experiments, the standard strain of *Escherichia coli* ATCC 25922 was used.

#### 2.3. Polymerase Chain Reaction (PCR) Detection of Carbapenemase Genes

To perform the PCR, 24-hour cultures of the bacteria were collected from a plate containing Mueller-Hinton agar in a loop size, then made up to a 200 ml sterilized water suspension and boiled for 10 minutes. After centrifugation, the conducted supernatant was used as a template DNA for PCR. Duplication was performed using a thermocycler machine. Traceable genes included bla*OXA*-51, bla*OXA*-58, bla*OXA*-23, bla*OXA*-24, and ISA-ba1 genes. The primers which were used in this study are written down in Table 1, as well as the reaction conditions as prescribed on the pertinent documentation.

#### 2.4. Pulsed Field Gel Electrophoresis (PFGE)

To perform PFGE, isolates of patients were cultured on TSA agar for 18 to 24 hours and incubated at 37°C and used to prepare the DNA and the plug according to the standard protocol. To prepare the plug, 400 µL of each microbial suspension was poured into a microtube and 400 µL of LMP agarose was added (agarose dissolved in TE buffer). Also, for each sample, 20 ng / ml were added to each micro tube and placed into the plug molding unit.

The samples were kept at room temperature for 5-15 minutes and then kept in the refrigerator for 10 minutes. Af-

### Table 1. Sequence of primers for resistance to carbapenems genes and expected PCR product length.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Fragment Length(bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bla<em>OXA</em>-51-like</td>
<td>5’-TAA TGC TTT GAT CGG CCT TG-3’</td>
<td>353</td>
<td>[20]</td>
</tr>
<tr>
<td>Bla<em>OXA</em>-23-like</td>
<td>5’-TGG ATT GCA CTT CAT CTT GG-3’</td>
<td>501</td>
<td>[20]</td>
</tr>
<tr>
<td>Bla<em>OXA</em>-24-like</td>
<td>5’-GAT CGG ATT GGA GAA CCA GA-3’</td>
<td>246</td>
<td>[20]</td>
</tr>
<tr>
<td>Bla<em>OXA</em>-26-like</td>
<td>5’-ATT TCT GAC CGC ATT TCC AT-3’</td>
<td>559</td>
<td>[20]</td>
</tr>
<tr>
<td>Bla<em>OXA</em>-38-like</td>
<td>5’-GAT TAG TTG GCC CCC TTA AA-3’</td>
<td>451</td>
<td>(21)</td>
</tr>
<tr>
<td>ISAba1</td>
<td>5’-CATTGGCATTAAACTGAGGAGAAAA-3’</td>
<td>451</td>
<td>(21)</td>
</tr>
</tbody>
</table>

*Note: The Patient samples were cultured on TSA agar for 18 to 24 hours and inbuated at 37°C. To perform PFGE, isolates of patients were cultured on TSA agar for 18 to 24 hours and inbuated at 37°C. The samples were kept at room temperature for 5-15 minutes and then kept in the refrigerator for 10 minutes. After centrifugation, the conducted supernatant was used as a template DNA for PCR. Duplication was performed using a thermocycler machine. Traceable genes included bla*OXA*-51, bla*OXA*-58, bla*OXA*-23, bla*OXA*-24, and ISA-ba1 genes. The primers which were used in this study are written down in Table 1, as well as the reaction conditions as prescribed on the pertinent documentation.*
Table 2. Percentage of phenotypic resistance of different antibiotics of *Acinetobacter baumannii*.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Resistant Strains N (%)</th>
<th>Intermediate Strains N (%)</th>
<th>Sensitive Strains N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefotaxime</td>
<td>48(96%)</td>
<td>1(2%)</td>
<td>1(2%)</td>
</tr>
<tr>
<td>Colistin</td>
<td>3(6%)</td>
<td>0(0%)</td>
<td>47(94%)</td>
</tr>
<tr>
<td>Meropenem</td>
<td>45(90%)</td>
<td>2(4%)</td>
<td>3(6%)</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>0(0%)</td>
<td>0(0%)</td>
<td>50(100%)</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>49(98%)</td>
<td>0(0%)</td>
<td>1(2%)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>43(86%)</td>
<td>3(6%)</td>
<td>4(8%)</td>
</tr>
<tr>
<td>Cefepime</td>
<td>45(90%)</td>
<td>1(2%)</td>
<td>4(8%)</td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>43(86%)</td>
<td>3(6%)</td>
<td>4(8%)</td>
</tr>
<tr>
<td>Ampicillin-sulbactam</td>
<td>50(100%)</td>
<td>0(0%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>17(34%)</td>
<td>17(34%)</td>
<td>16(32%)</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>43(86%)</td>
<td>4(8%)</td>
<td>3(6%)</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>48(96%)</td>
<td>1(2%)</td>
<td>1(2%)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>47(94%)</td>
<td>1(2%)</td>
<td>2(4%)</td>
</tr>
</tbody>
</table>

**Fig. (1).** Prevalence of antibiotics resistance among *Acinetobacter baumannii* isolates with 95% confidence interval.

After transferring these plugs into the test tube, lysis buffer was applied for 1.5 to 2 hours inside the shaker incubator at 55°C. Then each plug was washed twice with distilled water and 4 times with TE buffer. At the last wash, after emptying the TE buffer into the tube containing the plug, fresh buffer was added and the samples were placed at a temperature of 4-8°C. In the next step, for enzyme digestion and endonuclease genomic DNA fragmentation, Apal enzyme was used according to the protocol, the enzyme digestion was as follows. In the first step, the plugs were first mixed with 180cc distilled water and 20 µl of enzyme buffer (10X) and incubated microtubes for 15 minutes at room temperature, and again the plugs were transferred to another microtube, and were mixed with 175 µl of distilled water, 20 µl, 10X buffer and 5µl of the Apal enzyme and incubated for 5 hours at 25°C in a shaker incubator. Electrophoresis was performed in 1% agarose gel in Chef Drii in TBE 0.5X buffer with an initial switching program of 1 minute and 35 seconds, with a final switching time of 35 seconds, and a runtime 19 hours at 6 volts/cm. In the next step, the gel was stained in Ethidium bromide, washed with distilled water, and then analysed in a gel doc machine. Finally, the dendrogram pattern was determined and the relation of the samples clonal to each other was determined using Gel comparison software.
2.5. Statistical Analysis

Data were collected on standard forms, entered into an Excel database and analyzed by using the SPSS software version 20.0.

3. RESULTS

3.1. Clinical Isolates

Fifty isolates were identified as *Acinetobacter baumannii*, by standard biochemical tests.

3.2. Antimicrobial Resistance of *Acinetobacter baumannii* strains

The highest antibiotic resistance of *Acinetobacter baumannii* strains was respectively ampicillin sulbactam (100%), aztreonam (98%), cefotaxime and ceftriaxone (96%), ciprofloxacin (94%), cefepime and meropenem (90%), tetracycline, tobramycin and Trimethoprim-Sulfamethoxazole (86%) and amikacin (44%), the highest antibiotic susceptibility was polymyxin B (100%) and colistin (94%), respectively. The results of the phenotypic resistance study of *Acinetobacter baumannii* strains are shown in Table 2 and Fig. (1).

3.3. PCR Amplification for Detection of *bla*OXA-51, *bla*OXA-58, *bla*OXA-23, *bla*OXA-24, and ISAba1 genes

*Acinetobacter baumannii* distribution resistance genes in clinical strains are demonstrated in Table 3. All *Acinetobacter baumannii* strains contained the *bla*OXA-51-like gene.

3.4. Results of PFGE Method

In this study, all 50 strains of PFGE were typed. The results were analyzed using Gel comparison software. Based on an 80% cut off, 34 different patterns of different pulsating patterns were observed. Of the 34 patterns, 27 patterns contained more than one *Acinetobacter baumannii* strain, and 23 patterns had only one strain.

In this study, the highest frequency was related to patterns of 3,4,5,6,7, with 5 strains, and the lowest frequency of

<table>
<thead>
<tr>
<th>Genes</th>
<th>Resistant Strains N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>bla</em>OXA-23-like</td>
<td>44(88%)</td>
</tr>
<tr>
<td><em>bla</em>OXA-24-like</td>
<td>32(64%)</td>
</tr>
<tr>
<td><em>bla</em>OXA-51-like</td>
<td>50(100%)</td>
</tr>
<tr>
<td><em>bla</em>OXA-58-like</td>
<td>32(64%)</td>
</tr>
<tr>
<td>ISAba1</td>
<td>43(86%)</td>
</tr>
</tbody>
</table>

Fig. (2). *Acinetobacter baumannii* strain dendrogram with Gel compare software.
strains 1,2,8,9,10,12,14,15,17,18,20, 21,22,24,25,27,30,35, 36,37,38,45,48 with one strain. The dual strains included [46 and 47], [39 and 50], [43 and 49], [23 and 42], [26 and 28], [34 and 40], [41, 44] and [31 and 29] and triple strains [13, 16 and 19 and 11, 32 and 33]. The results of the PFGE study of strains are shown in Fig. (2).

4. DISCUSSION

Acinetobacter baumannii has been responsible for the spread of many hospital infections in recent decades. The bacterium is scattered throughout the world and causes many problems in humans [1, 22-25]. Previous studies have shown that front-line treatment for Acinetobacter baumannii infections includes amikacin, carbapenems, ceftazidime and quinolones. Among these, resistant strains to carbapenems have turned out to be a huge problem in the world, and are considered as a worldwide problem, since the treatment of these strains is very difficult [26-28]. Unfortunately, no data is available on the antibiotic resistance of Khorramabadi city isolates of Acinetobacter baumannii or on the spreading of genes involved in resistance to carbapenems.

In our study, all Acinetobacter baumanii strains were resistant to Ampicillin Sulbactam, which was similar to the findings of Wang et al., 2003 [29]. The findings of this study showed that the resistance to meropenem (a member of the carbapenem family) was about 90%, contrary to the Hujer study in 2003, which reported resistance to carbapenems of only 20% [30]. This contrast is related to the difference in antibiotic use in different regions.

In the study of Acinetobacter baumannii antibiotic resistance by Simhon, the resistance to imipenem increased from 1.9% in 1990 to 35.9% in 2000 [31]. Similarly, studies such as Talebi Taher in Tehran 2010-2011 (percentage resistance to imipenem was 97%), Zarrilli and Szczepan in 2013 (94.99%), indicate that resistance to carbapenems is increasing worldwide [32-34]. This study is similar to the study of Seng Stock and colleagues in the United States, of which more than 98% of the groups were resistant to azatrimion [35]. The results of this study, and its comparison with similar studies indicate that resistance to Acinetobacter baumanii is constantly changing, and can cause serious concerns in the field of treatment. Differences in the clinical samples studied, the timing of the study and treatment strategies in each geographic region may be responsible for the differences in the results of different studies.

The results of this study, as reported by Saeed Shoja (2016) in Alhaz, Bagheri-Joushagani (2016) in Kashan and Azimi in Tehran (2013), were 100% resistant to the blaOXA-51 gene [36-38]. In a study conducted by Shahchegrahi et al. in Tehran in 2011 on 100 samples, the percentage of resistance to the blaOXA-51 genome was 94% and blaOXA-23 84% [39]. In the study by Karmostaji in Tehran in 2013, the distributions of blaOXA-51, blaOXA-23, blaOXA-24, blaOXA-58 resistance genes were 93.3%, 81.3%, 8.1%, and 0.81%, respectively [40]. In the study by Azizi in 2015, in 65 samples, blaOXA-51 and blaOXA-23 genes were found in 100% of samples, blaOXA-24 in 29 samples (44%) and blaOXA-58 in no samples [41]. In a study conducted by Bagheri-Joushagani in 2016, the percentage of resistance to blaOXA-23 and blaOXA-24 was 90%, ISAba1, and blaOXA-51 were 100% and blaOXA-58 were reported as zero samples [36]. In a study conducted by Najar Piraye in Bandar Abbas, Iran in 2014, 78.9% of the samples had OXA-23 resistance gene, 8.8% had blaOXA-24 gene and 1.7% had blaOXA-58 gene [42]. In the study by Rabah Bakour in Algeria in 2015, blaOXA-51, blaOXA-23 and blaOXA-24 carbapenemase genes were detected in 100%, 67.02% and 20.21% of isolates, respectively [43]. In a study conducted by We, A Gebrey in the USA in 2009 on 83 samples, blaOXA-23 in 13% and IS-Ab1 in 79.5% of clinical isolates was reported [44]. The highest existence of resistance was in the blaOXA-51, blaOXA-23 and IS-Ab1 genes, and the lowest percentage in the blaOXA-58 gene. These are the results of various studies, which are in agreement with our findings. The difference in the prevalence of carbapenemase genes in different regions is due to differences in the pattern of use of beta-lactam and carbapenem antibiotics; so increasing the resistance to beta-lactams, due to the presence of plasmids, also causes resistance to other antibiotics.

In a study by Lin MF et al. in 2011 in China, 11 pulsotypes were obtained from 23 Acinetobacter baumannii isolates by the PFGE technique, which named them A to K respectively [45]. Zarrilli, 2004, in Italy, in a genotypic analysis of the Acinetobacter baumannii strains by PFGE from 131 patients revealed 9 distinct PFGE patterns, of which seven had only one strain [46]. Shahchegrahi et al. 2010, in Iran, acquired six types of 29 analyzed isolates, with type A being the most common type that included 19 isolates. The types named as B and C each were four and three isolates respectively, and the rest of D to F each contained one isolate [39]. In the present study of 50 samples, 33 different pulsotype was found, with the highest number of samples in 3, 4,5,6,7,5 strains. The genomic pattern obtained shows the different origin of the isolates. And as indicated in the results section, 23 pulsotypes had one strain and 11 pulsotypes had more than one strain.

CONCLUSION

Due to the high resistance of Acinetobacter baumannii and its wide distribution, as well as its ability to transfer resistance genes through moving genetic factors among important bacteria in hospitals, effective methods of hospital disinfection and sanitation observation are required. In addition, the use of broad-spectrum antibiotics should be reduced. Information from strain typing is, in fact, complementary to widespread epidemiological research, and its results, along with other phenotypic methods, such as antibiogram, is valuable in a limited time and space. As PFGE is directly related to the genetic sequencing of strains, it can be typed and replicated with good results and diagnostic power, and is nowadays a superior technique in tracing strain origin.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.
CONSENT FOR PUBLICATION
Not applicable.

AVAILABILITY OF DATA AND MATERIALS
Not applicable.

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CONFLICT OF INTEREST
The authors confirm that this article content has no conflict of interest.

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Molecular Detection of Carbapenem Resistance in Acinetobacter Baumannii


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