Molecular identification of TEM and SHV extended spectrum β-lactamase in clinical isolates of Acinetobacter baumannii from Tehran hospitals

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Abstract
Multidrug resistance (MDR) Acinetobacter baumannii, producing the extended-spectrum β-lactamases (ESBL) is one of the major concerns in hospitals. According to the infection reports caused by A.baumannii in Iran, the aim of this study is to identify the genes encoding the β-lactamases TEM and SHV in A.baumannii isolates in Tehran hospitals using PCR method. A total number of 200 clinical isolates A. baumannii were diagnosed at first using culture methods and biochemical tests followed by antibiogram using 16 different antibiotics by disk diffusion method based on CLSI guidelines. The ESBL was determined by double disk synergy test (DDST) and the results were verified using combined disk method. PCR for determining the prevalence of genes was performed using specific primers for all isolates. There was a high antibiotic resistance among the isolates with a high MDR rate of 82.5% and 51% strains were producing ESBL. The minimum inhibitory concentration (MIC) rate of cefepime and ceftazidime in the isolates were 8–128 μg/ml. The prevalence of TEM and SHV were respectively 56% and 63%. These results indicate the necessity of a monitoring program to timely identify these strains to prevent the resistance spread and increase efficient antibiotics usage.

Keywords: Acinetobacter baumannii, MDR, extended-spectrum β-lactamases, TEM, SHV

1 Introduction
Genus Acinetobacter includes 25 species, among which A. baumannii in human, is the most important one [1]. A. baumannii is a gram-negative, pleomorphic, aerobic, nonmotile, catalase-positive and oxidase-negative bacillus that exists in the natural environment (water and soil) and typically is isolated from hospital setting, respiratory secretions, wounds and urine of patients [2]. This organism is responsible for 10.2% of infections caused by gram-negative bacteria, in America and Europe [3].
This organism usually causes infections in people with compromised immune systems, especially in intensive care unit (ICU) patients who typically have invasive equipments, such as catheters and ventilators which increase the chance of *A. baumannii* infection. These infections include ventilator associated pneumonia (VAP), meningitis, septicemia, urinary tract infection (UTI), respiratory tract and wound infection. Therefore, timely diagnosis and treatment of the infections caused by this organism is extremely important [2, 3].

Since β-lactam antibiotics were used many years ago, thus β-lactamases have evolved along with them [4] *Acinetobacter* species has a wide range of β-lactamases, hydrolyzing the penicillin, cephalosporins and carbapenem [4, 5]. Today more than 300 types of β-lactamase have been identified that are divided into groups based on the structure and substrate they affect. ESBLs belong to the 2be group according to the Bush-Jacoby-Medeiros classification which are produced by the initial mutation and amino acid substitutions of early β-lactamases. ESBLs have altered enzymatic activity from their progenitors. Furthermore they have the ability to hydrolyze penicillins, first and second generation of cephalosporins. Adding that, they can also hydrolyze the third generation of cephalosporins and monobactams. The most prominent members of this group are TEM and SHV enzymes [6]. The main challenge in *A. baumannii* pathogenesis is unity, connection, expression and regulation of genetic elements that contribute to the MDR phenotype [4]. For example, the presence of OXA and ESBLs genes such as TEM and SHV lead to resistance to almost all β-lactams antibiotics, including carbapenems [4, 7].

There are frequent reports of these bacteria pathogenesis in Iran [5]. However, there is a palpable lack of applied studies which have investigated β-lactamases encoding genes among the *A. baumannii* isolated from the patients. With regards to the importance of identifying phenotypically and genotypically resistance, the aim of this study was to recognize the genes encoding the β-lactamase (SHV and TEM) and their role in resistance and nosocomial infections in patients of Tehran hospitals by using uniplex PCR. Furthermore the drug resistance patterns of these isolates to 16 different antibiotics were calculated so that the ESBL positivity and MIC were determined.

### 2 Materials and Methods

In this study which was done between March 2012 to April 2013, 850 samples were obtained from urine, blood, skin, wound and tracheal samples isolated from the respiratory tract of patients in nine hospitals in Tehran (Baqiatallah, Milad, Shariati, Imam Khumeini, Luqman, Shohada, Khatam-ol Anbia, Talqani, Mofid).

First, clinical samples were cultured on MacConkey agar and blood agar media (Merck, Germany) and they were incubated for 24 hours at 37°C. In order to see the growth, the gram staining was performed. After detecting the gram-negative coccobacilli, the oxidase test was implemented. In the next step, using standard biochemical tests and growth at 37°C and 42°C, the ultimate identification among *Acinetobacter* species was concluded [5]. Among the 850 suspected cases, 200 isolates were identified as *A. baumannii*.

To determine the antibiotic susceptibility of the isolates, the disk diffusion method (Kirby-Bauer) was used. The colony suspension from each overnight cultures was prepared and it was compared with the turbidity of the 0.5 McFarland standard. Accordingly, the noted was then cultured on the Mueller-Hinton agar medium. The antibiotic disks (MAST, England) containing aztreonam (30 μg), ampicillin-sulbactam (20 μg), ceftazidime (30 μg), ceftriaxone (30 μg), amikacin (30 μg), colistin (110 μg), cefotaxime (30 μg), imipenem (10 μg), piperacillin-tazobactam (110 μg), tobramycin (10 μg), cefepime (30 μg), gentamicin (12 μg), meropenem (10 μg), tetracycline, (30 μg) polymyxin B (300 μg) and piperacillin (100 μg) were placed on the media and they were incubated overnight at 37°C. Furthermore, the subsequent inhibition zone diameter was measured according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [8]. Following the results, the isolates were divided into susceptible (S), intermediate (I) and resistant (R) groups. For quality control test, *Escherichia coli* ATCC 25922 was used. MDR is defined as...
simultaneously resistant to three or more than three classes of antibiotics, including extended-spectrum cephalosporins (ceftazidime and cefepime), quinolones (ciprofloxacin), aminoglycosides (amikacin, tobramycin and gentamicin), the combination of β-lactamase and β-lactamase inhibitors (ampicillin-sulbactam) and the carbapenems (imipenem and meropenem). The determination of cefepime and ceftadizime MICs was performed by the agar-dilution method according to the CLSI guidelines [8]. In order to determine the production of ESBL, the DDST was used. Four disks of ceftriaxone (30 μg), ceftazidime (30 μg), cefotaxime (30 μg) and cefepime (30 μg) were put around an amoxicillin-clavulanate disk (20 μg/10 μg) with a regular distance of 15 mm from the central disk. In the case of increase and or irregularity of the inhibition zone which exists between cephalosporins and amoxicillin-clavulanate disk, the isolates was considered as ESBL positive (9). ESBL production was confirmed by combined disk method using cefotaxime (30 μg) and ceftazidime (30 μg) antibiotic disks with and without clavunalic acid (10 μg) on Mueller-Hinton agar as recommended by CLSI. Accordingly, the plates were incubated overnight at 37°C, a ≥5 mm increase in the inhibition zone around combined disks were considered ESBL-positive, the other remaining organisms were considered ESBL-negative [9]. For quality control test, the following organisms were used: E. coli ATCC 25922 as negative control and A. baumannii ATCC 25922 as positive control. Data analysis was performed using SPSS software version 17 and frequencies were calculated and expressed as percentage by using descriptive statistics.

Genomic DNA of the isolates was extracted using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany). Primer sequences for amplification of blaTEM and blaSHV genes are listed in table 1. Amplification reactions were run under the following conditions. The initial denaturation at 95°C for 3 minutes under 35 cycles followed by the denaturation temperature at 95°C for 45 seconds and a subsequent annealing at 56.6°C for 30 seconds for SHV primer and 58.4°C for TEM primer for 30 seconds. Extension temperature was 72°C for 1 minute, and final extension was 72°C for 3 minutes. After the reaction, the products were electrophoresed for 1 hour with 80 voltages on %1.5 agarose gel stained with SYBR green solution. Furthermore the bands were detected by gel document. In order to determine the size of the obtained bands, 100bp DNA ladder (Sinacolon Company) and positive control containing the target genes were used and ultimately some of the PCR products were sequenced. Similarity and alignments of both the nucleotide sequences were performed by the BLAST search and compared with DNA GenBank.

### Table 1: Primers used for amplification of ESBLs genes

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide Sequences</th>
<th>PCR length</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-F</td>
<td>TTTCTGTCGCCCTTATTCC</td>
<td>401bp</td>
</tr>
<tr>
<td>TEM-R</td>
<td>ATCGTTGTCAAGTAAGTTGG</td>
<td></td>
</tr>
<tr>
<td>SHV-F</td>
<td>CGCCTGTTATATCCCTCT</td>
<td>293bp</td>
</tr>
<tr>
<td>SHV-R</td>
<td>CGAGTAGCCACAGATCCT</td>
<td></td>
</tr>
</tbody>
</table>

### 3 Results

Among a total number of 242 *Acinetobacter* isolates from 850 samples, 200 isolates (82.7%) were identified as *A.baumannii* and 33 *A.lwoffii* (13.6%) and 9 (3.7%) were the other *Acinetobacter* species. 75 samples were obtained from the ICU, 18 from pediatrics care unit, 32 from burn unit, 40 from infectious diseases unit, 24 from emergency unit and 11 from surgical unit were isolated, among which the highest level was related to ICU. The *A. baumannii* prevalence rate in clinical samples is listed in chart 1. The prevalence rate of antibiotic resistance in *A.baumanii* strains is presented in table 2. The most resistance rate was related to aztreonam, cefepime, cefeteraxone, ceftazidim, cefotaxime. The least amount of resistance belonged to polymyxin B and no resistance was seen against colistin. The MDR rate was
determined 82.5%. The results of screening ESBL by DDST and combined disk method showed that 51% of isolates were producing ESBL. All ESBL-producing isolates were resistant to all β-lactams antibiotics. The MIC rate of cefepime was determined ≥64 μg/ml in 54.5% of the samples and >128 μg/ml in 58% of the samples against ceftazidime. Molecular analysis showed that 63% and 56% of isolates respectively carried SHV and TEM genes that could be observed on agarose gel electrophoresis at 293bp for SHV and 401bp for TEM genes (Figure 1). The most resistance rates in isolates carrying SHV genes respectively belonged to aztreonam, cefotaxime, ceftriaxone, cefepime, amikacin, imipenem and piperacillin-tazobactam. On the other hand for the isolates carrying the TEM gene the most resistance rate belonged to ceftriaxone, cefepime, ceftazidime, aztreonam, amikacin, piperacillin and imipenem.

![Chart 1: Distribution of the A.baumannii isolates among clinical samples](chart1.png)

**Table 2: Antibiotic resistance patterns among the A. baumannii isolates**

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Susceptible phenotype</th>
<th>Intermediate phenotype</th>
<th>Resistance phenotype</th>
<th>Rate of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftadizime</td>
<td>11</td>
<td>3</td>
<td>186</td>
<td>93%</td>
</tr>
<tr>
<td>Colistin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ampicillin-sulbactam</td>
<td>65</td>
<td>30</td>
<td>105</td>
<td>52.5%</td>
</tr>
<tr>
<td>Amikacin</td>
<td>20</td>
<td>8</td>
<td>172</td>
<td>86%</td>
</tr>
<tr>
<td>Piperacillin-tazobactam</td>
<td>35</td>
<td>5</td>
<td>160</td>
<td>80%</td>
</tr>
<tr>
<td>Imipenem</td>
<td>9</td>
<td>20</td>
<td>171</td>
<td>85.5%</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>2</td>
<td>5</td>
<td>193</td>
<td>96.5%</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>11</td>
<td>17</td>
<td>172</td>
<td>86%</td>
</tr>
<tr>
<td>Cefepime</td>
<td>2</td>
<td>1</td>
<td>197</td>
<td>98.5%</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>156</td>
<td>0</td>
<td>44</td>
<td>22%</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>44</td>
<td>10</td>
<td>146</td>
<td>73%</td>
</tr>
<tr>
<td>Cefotaxim</td>
<td>7</td>
<td>10</td>
<td>183</td>
<td>91.5%</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>30</td>
<td>12</td>
<td>158</td>
<td>79%</td>
</tr>
<tr>
<td>Tetracycllin</td>
<td>40</td>
<td>19</td>
<td>141</td>
<td>70.5%</td>
</tr>
<tr>
<td>Meropenem</td>
<td>17</td>
<td>19</td>
<td>164</td>
<td>82%</td>
</tr>
<tr>
<td>aztreonam</td>
<td>0</td>
<td>0</td>
<td>200</td>
<td>100%</td>
</tr>
</tbody>
</table>
Figure 1: PCR detection of \(\text{bla}_{\text{SHV}}\) and \(\text{bla}_{\text{TEM}}\) M: DNA size marker (100bp DNA ladder, SM#333), Lane 1: negative control, Lane 2-3: PCR products of the isolates containing \(\text{bla}_{\text{SHV}}\) (293 bp). Lane 4-5: PCR products of the isolates containing \(\text{bla}_{\text{TEM}}\) (401bp).

4 Discussion

One of the main concerns about \(A.\ baumannii\) in Iran within hospitals is its resistance to a wide range of antibiotics caused by the rampant overconsumption of antibiotics including extended-spectrum \(\beta\)-lactames and carbapenems as the latest treatment options [10]. Accurate detection of ESBL genes involved in resistance is important for monitoring, treatment and epidemiology studies to understand how they are transmitted in hospitals, because these strains are able to transfer resistance genes to other clinical strains as well [11]. Consequently, early detection of these strains is important in microbiology laboratories. Molecular methods such as PCR will determine which type of ESBL exists in the isolates [4]. In this study, the rate of phenotype ESBL production and TEM and SHV genes frequency, as well as the frequency of \(A.\ baumannii\) strains isolated from patients in Tehran hospitals were studied. In the present study, into most resistance rate observed in aztreonam (100%), cefepime (98.5%), ceftriaxone (96.5%), ceftazidime (93%), cefotaxime (91.5%) amikacin (86%), piperacillin (86%), imipenem (85.5%), meropenem (82%) and antibiotics such as polymyxin B and colistin were considered as the most effective drugs against \(A.\ baumannii\) strains. Our findings largely agree with results of Ayan et al. in 2003 who reported all the \(A.\ baumannii\) isolates were resistant to ceftiazoxone, cefepime, ceftazidime, piperacillin, piperacillin-tazobactam, gentamicin and aztreonam but resistance rate to amikacin and tobramycin were higher in this study [12]. In another study in Iran, Owlia et al in 2012 concluded that more than 90% of \(A.\ baumannii\) isolates were resistant to cefteriaxone, cefepime, ceftazidime, piperacillin, piperacillin-tazobactam, gentamicin and aztreonam but resistance rate to amikacin and tobramycin were higher in this study [12]. In another study in Iran, Owlia et al in 2012 concluded that more than 90% of \(A.\ baumannii\) isolates were resistant to aztreonam, ceftadizim, cefotaxime and similar to our study the reported resistance to imipenem was determined 85% with no reported colistin resistance. However, contrary to our study, Owlia et al. reported the resistance rate of tobramycin to be 58% [10]. Similar to our findings, Zhou et al. in 2007 reported high resistance rate to extended spectrum \(\beta\)-lactams, monobactams, carbapenems and aminoglycosids [13]. In the present study, the resistance rate against imipenem is in line with the findings presented by Ramoul et al. in 2013 (>80%) [14] and is in contrast with Hujer et al. in 2006 (20%) [15] and Rahbar et al. in 2010 (4.5%) [16]. The determined resistance rate of meropenem (82%) is in contrast with findings presented by Hujer et al. (25%) [15], but similar to the findings of Fazeli et al. in 2013 (80.95%) [17]. The above noted congruent findings may be due to the similar process of research. Furthermore, the varying findings can be construed due to using different types of samples, the type of antibiotic disks used and the variation in performing the antibiotic susceptibility test.
Rate of isolates producing ESBL phenotype was 51% that is compatible with the findings of Jazani et al. in 2010 and Yong et al. in 2003 that reported the prevalence rate of ESBL among A. baumannii isolates 48% and 54.6% respectively [18, 19]. However, our result is in contrast with the report of Sinha et al. in 2007 acknowledging 28% in the noted aspect [9]. This increase in prevalence rate of ESBL among A. baumannii in comparison to the previous studies conducted in Iran confirms the growing need for the treatment of the infected patients.

In a study conducted in 2007, Lim et al. reported the MIC rate against ceftazidim and cefepime >128 μg/ml and 64 μg/ml respectively in 27 strain of A.baumannii harboring ESBL genes [20] that is similar to our findings. The MIC rate in our study to cefepime and ceftazidim was respectively ≥64 μg/ml and >128 μg/ml in 54.5% and 58% of the samples. Our results are in contrast with the findings presented by Danes et al. in 2002 which reported the MIC rate of ceftazidim and cefepime ≥256 μg/ml and ≥32 μg/ml respectively [21]. The difference in findings may be due to an increase in resistant strains or existing varying in investigated samples.

The MDR rate varies in different countries due to the environmental factors and different antibiotic consumption patterns. Our finding in the present study (82.5%) is similar to the result of karbasizade et al. in 2012 that reported 85% MDR rate among 50 A.baumannii strains [22] and Hujer et al. (89%) [15]. However our conclusion is contrary to the findings presented by Smolykav et al. in 2003 which was reported to be 54% [23]. The variant findings may be associated with different geological settings, the time gap between the current study and previous ones as well as utilizing different antibiotics within the experiments.

Molecular analysis of isolates showed that 56% and 63% of isolates were respectively TEM and SHV positive genes. In a survey conducted by Zhong-nong et al. in China in 2006, it was found that TEM gene frequency among 39 isolates of A.baumannii was 33.3% [24]. In a study conducted by Endimiani et al. in 2007 reported that 31 isolates out of 470, were carrying the TEM-92 gene [25]. Jin et al. in 2009 reported that TEM positive samples among isolates were 81.5% that differed from our findings [26]. Dai et al. reported in 2010, among 39 isolates of A.baumannii, 2 isolates contained SHV gene and 15 isolates contained TEM gene [27]. Our findings is similar to Haung et al. in 2004 and Pooshaneh et al. in 2011 which confirmed that SHV genotype had a higher prevalence rate than TEM genotype [28, 29]. TEM gene prevalence in this study is similar to the result of Yin et al. in 2008, (61.3%) [30], and is in contrast with the results of Adams-Haduch et al. in 2008 (73.5%) [31] and Danes et al. (25%) [21]. SHV gene frequency in this study was in contrast with the report Taherikalani et al. study in 2012 which did not report any cases of these genes in the Acinetobacter isolates [32]. The observed contrast of the results in comparison with the studies conducted in other countries can be a cause of geographic distance which may have affected within variation of resistance pattern in several areas, the antibiotic consume pattern and bacterial species being tested. The high prevalence rate of the genes in this study indicates the increasing dissemination of ESBL encoding genes among A.baumannii which is caused by inappropriate use of antibiotics in Iran. However; resistance to extended-spectrum cephalosporins, penicillins, broad-spectrum antibiotics, meropenem and imipenem must be controlled.

In this study the highest resistance rate to cephalosporins, monobactam and extended-spectrum penicillin antibiotics (piperacillin/tazobactam) was associated with strains containing TEM and SHV genes. The production rate of ESBL in this case was identified 51%. Considering the fact that the ESBL-producing organisms have shown a high resistance to other classes of antibiotics especially carbapenems and aminoglycosides, it can be concluded that the other elements of resistance, like carbapenem-hydrolyzing-β-lactamases, aminoglycoside modifying enzymes, changes in porins, and over expression of efflux pumps might be considered as the other resistance parameters. According to the growing and threatening danger of these bacteria, the rapid identification and detection of this strain have an important role in preventing their spread. Furthermore, these findings illuminate the necessity of review in antimicrobial therapy, use of
proper antibiotics and the utilization of new antimicrobial elements in order to treat the infections resulting from these bacteria.

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http://dx.doi.org/10.1016/S0195-6701(03)00046-X


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