Limited genetic diversity and extensive antimicrobial resistance in clinical isolates of Acinetobacter baumannii in north-east Iran

Hadi Farsiani,1,2,3† Arman Mosavat,1,2,3† Saman Soleimanpour,1,2,3 Mahbobeh Naderi Nasab,2,3 Himen Salimizand,1,2,3 Saeid Amel Jamehdar,2,3 Kia Rash Ghazvini,2,3 Ehsan Aryan2,3 and Ali-Asghar Baghani1,2,3

Correspondence
Saman Soleimanpour
soleimanpour.saman@yahoo.com
or soleimanpours881@mums.ac.ir

1Student Research Committee, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
2Antimicrobial Resistance Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
3Department of Microbiology and Virology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

This study determined the mechanisms and patterns of antimicrobial resistance among the isolates obtained from different wards of a teaching hospital in the city of Mashhad in north-east Iran. Between January 2012 and the end of June 2012, 36 isolates of Acinetobacter baumannii were collected from different wards of Ghaem Hospital. Antimicrobial susceptibility testing and epsilometer testing (E-test) were performed. The genetic resistance determinants of A, B and D classes of \( \beta \)-lactamases, aminoglycoside modifying enzymes (AMEs), efflux pumps and IS\( \text{Aba}1 \) elements were assessed by PCR. Repetitive extragenic palindromic element (REP)-PCR was performed to find the genetic relatedness of the isolates. Colistin was the most effective antibiotic of those tested, where all isolates were susceptible. E-test results revealed high rates of resistance to imipenem, ceftazidime and ciprofloxacin. The majority of isolates (97 %) were multidrug-resistant. OXA-51, OXA-23 and tetB genes were detected in all isolates, but OXA-58, IMP and tetA were not detected. The prevalence of OXA-24, \( \text{bla}_{\text{TEM}} \), \( \text{bla}_{\text{ADC}} \), \( \text{bla}_{\text{VIM}} \) and \( \text{adeB} \) were 64, 95, 61, 64 and 86 %, respectively. IS\( \text{Aba}1 \) was found to be inserted into the 5′ end of OXA-23 in 35 isolates (97 %). Of the AMEs, \( \text{aadA}1 \) (89 %) was the most prevalent, followed by \( \text{aphA}1 \) (75 %). The band patterns reproduced by REP-PCR showed that 34 out of 36 isolates belonged to one clone and two singletons were identified. The results confirmed that refractory A. baumannii isolates were widely distributed and warned the hospital infection control team to exert strict measures to control the infection. An urgent surveillance system should be implemented.

INTRODUCTION

Acinetobacter baumannii is an opportunistic microorganism that is of concern in hospital-acquired infections (Dijkshoorn et al., 2007). This pathogen mainly infects patients in intensive care units (ICUs) and was first reported in the USA in 1992 (Go et al., 1994); many hospital outbreaks have subsequently been reported worldwide (Erdem et al., 2014). Its propensity to acquire resistance genes allows A. baumannii to survive under selective antibiotic pressure (Visca et al., 2011). Antibiotics considered for effective treatment of A. baumannii infections include carbapenems, cephalosporins, aminoglycosides, polymyxins (colistin) and tigecycline, solely and in combination; however, refractory isolates have arisen with the exertion of resistance genes (Rahal, 2006). Resistance to carbapenems is mainly mediated by carbapenem-hydrolysing \( \beta \)-lactamases (OXA-23, OXA-24/40, OXA-51 and OXA-58) and metallo-\( \beta \)-lactamases (MBLs; IMP, VIM, GIM, SIM and SPM) (Higgins et al., 2015).
Resistance to tetracycline occurs in response to active efflux pump (tetA, tetB and tetH) and ribosomal protection (tetM) (Guardabassi et al., 2000). Aminoglycoside resistance genes confer resistance to this class of antibiotics by different modifying enzymes, including phosphotransferases (aphA1 and aphA6), nucleotidyldtransferases (aadA1 and aadB) and acetyltransferase (aacC1) (Ramirez & Tolmasky, 2010). In addition, intrinsic resistance to all these antibiotics could occur by means of general AdeABC efflux pumps (adeB) (Yoon et al., 2013). To control MDR isolates and eradicate refractory species from hospital settings, the epidemiology of the resistance determinants are of value.

Methodology

Study population and identification of bacterial isolates

From January to June 2012, 36 non-duplicate A. baumannii isolates were collected at Ghaem University Hospital. These strains were isolated from clinical samples (wound, tracheal aspirate, urine, oedema discharge, blood, cerebrospinal fluid (CSF)). The samples were obtained from patients hospitalized in the intensive care unit (ICU; n = 14), surgery (SUR; n = 7), newborn intensive care unit (NICU; n = 12), paediatrics (PED; n = 1) and internal medicine (IM; n = 2) wards in the 1000-bed Ghaem Hospital. Bacterial characterisation was done by biochemical reactions, oxidase test and oxidation/fermentation tests (Mahon et al., 2011) and ability for growth at 42 °C (Bouvet & Grimont, 1987). The isolates were stored at −70 °C until further investigation.

Antibacterial susceptibility testing

Susceptibility testing for 14 antibiotics was carried out using the Kirby–Bauer disc diffusion method, and the results were interpreted according to the 2012 Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012). The antibiotic discs contained ciprofloxacin (5 μg), amikacin (30 μg), gentamicin (10 μg), tobramycin (10 μg), trimethoprim/sulfamethoxazole (1.25 μg/23.75 μg), tetracycline (30 μg), doxycycline (30 μg), imipenem (10 μg), meropenem (10 μg), cefazidime (30 μg), cefotaxime (30 μg), piperacillin (100 μg) and cefepime (30 μg) (MAST Group). MIC testing was further evaluated for cefazidime, imipenem, ciprofloxacin and colistin using E-test strips (Lonza) according to the manufacturer’s instructions. These antibiotics were selected for detection of MDR strains. The strains were considered to be MDR and resistant to at least one agent in three or more antimicrobial classes (Magiorakos et al., 2012). Escherichia coli ATCC 25922 and A. baumannii ATCC 19606<sup>T</sup> were used as quality control strains.

Genomic experiments

DNA extraction. In order to obtain genomic DNA, 36 A. baumannii strains were grown overnight at 37 °C on Mueller–Hinton (Merck, Germany) agar plates until the recovery of fresh culture from each isolate. A loopful of each pure bacterium was then suspended in 300 μl sterile distilled water and boiled for 10 min. After 5 min of centrifugation (10 000 g), the supernatant was used as the DNA template for PCR amplifications (Yan et al., 2010).

Molecular detection of resistance genes. DNA of the isolates was prepared by boiling. The resistance determinant genes were oxacillinases (OXA-23, OXA-24/40 and OXA-51), ESBL (bla<sub>TEM</sub>), cefalosporinase (bla<sub>ADC</sub>), MBLs (IMP, VIM), aminoglycoside modifying enzymes (AMEs; aphA1, aphA6, aadA1, aadB and aacC1), general AdeABC efflux pumps (adeB) and specific active efflux pumps (tetA and tetB) and were detected by PCR (Fig. 1) through specific primers (Table 1). A combination of forward primers of ISAba1 and bla<sub>OXA-families</sub> were assessed to ascertain probable overexpression of bla<sub>OXA-families</sub> from the strong promoter provided by the ISAba1 element (Turton et al., 2006a). Standard strains for PCR tests were not available; therefore, the first amplified PCR products of the expected size in the electrophoresis gel were sequenced and considered as positive controls.

Clonal analysis and typing. Typing of isolates was performed by PCR amplification of repetitive extragenic palindromic elements (REP-PCR). PCR amplification was performed in a reaction mix containing REPI and REP2 primers (50 pmol μl<sup>−1</sup>), dNTPs mixture (0.2 mM), MgCl₂ (3 mM), 1 unit Taq DNA polymerase (Thermo Scientific), 1 × PCR buffer and bacterial DNA template (2 μl) in a final volume of 25 μl. The following temperature profile was used: initial denaturation at 95 °C for 10 min, 30 cycles of denaturation at 95 °C for 1 min, annealing at 45 °C for 1 min, extension at 72 °C for 2 min and a final extension at 72 °C for 16 min (Bou et al., 2000). PCR products were run in 2% agarose gel and electrophoresed through TBE 0.5 × buffer. Bands were seen after ethidium bromide staining and UV visualization. Band patterns were analysed using the
ceftriaxone, cefotaxime, cefepime, imipenem, meropenem, and 2.7% (1/36), respectively. It was found that 97% All strains (except strain no. 22) were MDR.

44.4% of the hospitalized patients were male and 55.6% (39%), wounds (n=4; 11.1%), CSF (n=2; 5.5%), urine (n=1; 2.7%) and oedema discharge (n=1; 2.7%). Overall, 44.4% of the hospitalized patients were male and 55.6% were female.

**Characteristics of the study population**

Of the 36 A. baumannii isolates collected, 26 (72.2%) were isolated from ICU, seven (19.4%) from SUR, two (5.6%) from IM and one (2.8%) from PED. The isolates were recovered from tracheal aspirate (n=14; 39%), blood (n=14; 39%), wounds (n=4; 11.1%), CSF (n=2; 5.5%), urine (n=1; 2.7%) and oedema discharge (n=1; 2.7%). Overall, 44.4% of the hospitalized patients were male and 55.6% were female.

**Antibacterial susceptibility testing**

All strains (except strain no. 22) were MDR A. baumannii. The rate of susceptibility to doxycycline, tetracycline, tobramycin gentamicin, ciprofloxacin, amikacin and amikacin were 50 (18/36), 2.7 (1/36), 19.4 (7/36), 5.5 (2/36), 8.3% (3/36) and 2.7% (1/36), respectively. It was found that 97% (35/36) of isolates were completely resistant to ceftazidime, ceftriaxone, cefotaxime, cephalosporins, imipenem, meropenem, piperacillin and ciprofloxacin. The results of disc diffusion assays indicated that 10 isolates (27.7%) were sensitive to aminoglycosides (tobramycin, gentamicin and amikacin).

It was alarming to find that six isolates (16.6%) were resistant to all antibiotics. MIC by E-test strips revealed that the most efficient antibacterial agent was colistin, to which all isolates were susceptible; however, 97% of isolates (except strain no. 22) were resistant to imipenem, ciprofloxacin and ceftazidime (Fig. 1).

**Molecular detection of resistance genes**

All isolates were assessed to find the genetic profiles of the resistance genes, including ß-lactamases (oxacillinases, MBLs and ESBLs), AMEs, ISAba1 element, and adeB, tetA and tetB efflux pumps. OXA-51, OXA-23 and tetB were detected in all isolates and OXA-58, IMP and tetA were not detected. Interestingly, the OXA-23 carbapenemase-encoding gene was detected in all isolates of A. baumannii, but the OXA-58 carbapenemase-encoding gene was not found. The prevalence of OXA-24, blaTEM, blaADC and blaVIM genes was 64 (23/36), 95 (34/36), 61 (22/36) and 64 (23/36), respectively. The findings revealed that 31 isolates (86%) contained the adeB gene, and ISAba1 was detected in 97% (35/36) of isolates of A. baumannii. Moreover, ISAba1 was detected to be inserted into the 5’ end of OXA-23 in 35 isolates (97%). No ISAba1 was found upstream of the OXA-51 and OXA-24/40 genes.

The most prevalent AME genes were aadA1 (n=32; 89%), followed by aphA1 (n=27; 75%). The prevalence of aphA6, aadB and aacC1 genes was 38.8 (14/36), 25 (9/36) and 16.6% (6/36), respectively. Thirty-four strains (94.4%)
A total of 36 isolates were submitted for molecular typing by REP-PCR. REP-PCR fingerprinting analysis results classified the 36 isolates into three main profiles. The vast majority of A. baumannii isolates belonged to only one major clonal group. The band patterns indicated that 34 (94.4%) of 36 isolates belonged to one clone and two singletons were identified (Fig. 1). The main clone was distributed throughout the wards over the timeline of the study.

## DISCUSSION

A. baumannii is an emerging pathogen commonly found in hospitalized patients, especially those in ICU wards (Kim et al., 2014). Countrywide reports of outbreaks of MDR strains have increased in the past few years in Iran. Most outbreaks occurred in ICU wards; however, in Ghaem Hospital, resistant isolates have increased in all wards.

Carbapenems are considered to be the last resort for treatment of A. baumannii infections; however, the contribution of carbapenem hydrolysing enzymes (oxacillinases and MBLs) at Ghaem Hospital was observed. The results showed that all isolates of A. baumannii harboured the blaOXA-51 gene, which can be used as a marker for identification of the A. baumannii species (Turton et al., 2006b). In addition, ISAba1 was detected in 97% of

### Table 1. Primers sequences used

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer</th>
<th>Annealing (°C)</th>
<th>Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaTEM</td>
<td>F: 5′-GCGTCGCTATATGATGATGATGCTGAC-3′</td>
<td>52</td>
<td>390</td>
<td>Ellington et al. (2007)</td>
</tr>
<tr>
<td>blaTMP</td>
<td>F: 5′-GGATAGAGTCCATCATGAC-3′</td>
<td>54</td>
<td>242</td>
<td>This study</td>
</tr>
<tr>
<td>blaOXA-24</td>
<td>F: 5′-TTAGGTGGCGGTACTTGGGTC-3′</td>
<td>59</td>
<td>206</td>
<td>Nemea et al. (2004)</td>
</tr>
<tr>
<td>adeB</td>
<td>F: 5′-ATACAGCATCCAAGCGGCA-3′</td>
<td>52</td>
<td>105</td>
<td>Srinivasan et al. (2009)</td>
</tr>
<tr>
<td>aphA1</td>
<td>F: 5′-GCCGTCACTATGTTACACG-3′</td>
<td>51</td>
<td>623</td>
<td>Noppe-Leclercq et al. (1999)</td>
</tr>
<tr>
<td>aphA6</td>
<td>F: 5′-CGACATCAAATGAACTGCGA-3′</td>
<td>55</td>
<td>780</td>
<td>Hujer et al. (2006)</td>
</tr>
<tr>
<td>aacC1</td>
<td>F: 5′-TTAGGTGGCGGTACTTGGGTC-3′</td>
<td>64</td>
<td>421</td>
<td>Hujer et al. (2006)</td>
</tr>
<tr>
<td>aadB</td>
<td>F: 5′-ATACAGCATCCAAGCGGCA-3′</td>
<td>70</td>
<td>495</td>
<td>Hujer et al. (2006)</td>
</tr>
<tr>
<td>aadA1</td>
<td>F: 5′-GCCGTCACTATGTTACACG-3′</td>
<td>64</td>
<td>792</td>
<td>Hujer et al. (2006)</td>
</tr>
<tr>
<td>blaTEM-1</td>
<td>F: 5′-GCCGTCACTATGTTACACG-3′</td>
<td>65</td>
<td>310</td>
<td>Hujer et al. (2006)</td>
</tr>
<tr>
<td>blaADC</td>
<td>F: 5′-GCCGTCACTATGTTACACG-3′</td>
<td>50</td>
<td>1081</td>
<td>Hujer et al. (2006)</td>
</tr>
<tr>
<td>OXA-23</td>
<td>F: 5′-TTGTTGATGCCCGTATGCAA-3′</td>
<td>55</td>
<td>167</td>
<td>This study</td>
</tr>
<tr>
<td>OXA-51</td>
<td>F: 5′-GCCGTCACTATGTTACACG-3′</td>
<td>55</td>
<td>276</td>
<td>This study</td>
</tr>
<tr>
<td>REP-PCR</td>
<td>F: 5′-CGACATCAAATGAACTGCGA-3′</td>
<td>45</td>
<td>Variable</td>
<td>Bou et al. (2000)</td>
</tr>
</tbody>
</table>

had at least one AME gene, 15 isolates (41.6%) had three AME genes, 14 isolates (38.8%) had two, four isolates (11.1%) had one, two isolates (5.5%) had four and one isolate (2.7%) had no AME genes. Fig. 1 shows the prevalence of resistance determinants.

### Clonality and genotyping

A total of 36 isolates were submitted for molecular typing by REP-PCR. REP-PCR fingerprinting analysis results classified the 36 isolates into three main profiles. The vast majority of A. baumannii isolates belonged to only one major clonal group. The band patterns indicated that 34 (94.4%) of 36 isolates belonged to one clone and two singletons were identified (Fig. 1). The main clone was distributed throughout the wards over the timeline of the study.

DISCUSSION

A. baumannii is an emerging pathogen commonly found in hospitalized patients, especially those in ICU wards (Kim et al., 2014). Countrywide reports of outbreaks of MDR strains have increased in the past few years in Iran. Most outbreaks occurred in ICU wards; however, in Ghaem Hospital, resistant isolates have increased in all wards.

Carbapenems are considered to be the last resort for treatment of A. baumannii infections; however, the contribution of carbapenem hydrolysing enzymes (oxacillinases and MBLs) at Ghaem Hospital was observed. The results showed that all isolates of A. baumannii harboured the blaOXA-51 gene, which can be used as a marker for identification of the A. baumannii species (Turton et al., 2006b). In addition, ISAba1 was detected in 97% of
carbapenem-resistant isolates of A. baumannii. The prevalence of blaOXA-23 and blaOXA-24 genes in clinical isolates of A. baumannii was 100 and 64%, respectively. These figures were higher than the findings of Feizabadi et al. (2008), who reported a 25% prevalence of the blaOXA-23 gene and a 17% prevalence of the blaOXA-24 gene in clinical isolates of A. baumannii. Moreover, a study conducted on isolates recovered from burn ward and ICU patients in Tehran reported a low rate of ISAba1 (32.2%) and an median rate of blaOXA-23 (67%), which differs from the results of the present study (Nasrolahei et al., 2014). We also found that 64% of isolates were VIM-family MBL producers; however, there were non-MBL carrier isolates in the samples which were imipenem-resistant. A study carried out during the same period at Imam Reza Referral Center, located near Ghaem Hospital, did not detect MBLs (Salimizand et al., 2015).

The same testing in the present study on the A. baumannii samples from Ghaem Hospital found only one isolate (isolate no. 22) that did not harbour ISAba1. The remaining isolates showed a sharp band near 1.6 kb; sequencing of this band confirmed an ISAba1/OXA-23 arrangement. Isolates that were not MBL producers, but were carbapenem-resistant, could be ascribed to this arrangement. This finding should be evaluated by real-time PCR to determine which MBL or ISAba1/OXA-23 was selected by bacteria to combat antibiotic stress. Other studies have noted the contribution of ISAba1/OXA-23 for carbapenem resistance (Peymani et al., 2012; Salimizand et al., 2015).

Extended distribution of OXA-23 adjacent to transposable elements in Iranian A. baumannii isolates indicates a plasmid-borne transfer among strains of the species. More study is required to determine if this kind of carbapenem resistance is endemic throughout the country or clonal to specific provinces. Several reports from different centres that implicate the role of MBLs in carbapenem resistance should not be neglected. Shahcheraghi et al. (2011) reported A. baumannii SPM producers in Tehran medical centres. Moreover, Fallah et al. (2014) and Peymani et al. (2011) reported VIM and IMP MBL producers. It should be noted that the rate of VIM producers in Ghaem Hospital (76%) was the highest ever reported in Iran. Many reports from Iran discuss the prevalence of resistance genes in A. baumannii, but most were limited to a narrow spectrum of resistance profiles. For example, Salimizand et al. (2014) found a correlation between the presence of OXA-23 and imipenem resistance in carbapenem resistance isolates, but did not assess the reasons for this phenomenon.

Besides the oxacilinases in A. baumannii, ESBLs could result in high resistance to cephalosporin antibiotics (Shakibaie et al., 2012). Unfortunately, the TEM variant of ESBL was not studied in Iran (Asadollahi et al., 2012); however, comprehensive multi-country studies have revealed high rates of TEM distribution in A. baumannii isolates (Chaudhary, 2012). The TEM variant appears to be more prevalent in Iran than in other Middle Eastern countries since it was produced by almost all the samples in the Ghaem Hospital collection (95%) (Al-Agamy et al., 2014; Hujer et al., 2006).

Aminoglycosides with β-lactams is a combination therapy that has been widely prescribed for A. baumannii infection (Karaioskos & Giamarellou, 2014). Aminoglycoside resistance has not been studied as thoroughly as carbapenem resistance in A. baumannii in Iran, but some studies have reported that aphA6 and aacC1 were the most prevalent aminoglycoside modifiers (Aghazadeh et al., 2013; Asadollahi et al., 2011; Aliakbarzade et al., 2014). This is not in accordance with results of the present study that found aadA1 followed by aphA1 to be the most widely distributed AMEs in the isolates. Antibiotic susceptibility screening revealed that 97% (35/36) of isolates were fully resistant at one at least one from among gentamicin, tobramycin or amikacin and at least one resistance gene was detected in all isolates. Investigation of other resistance mechanisms to aminoglycosides, such as alteration of the ribosome-binding site, reduced uptake and expression of AdeABC efflux pumps, can help determine the correct correlation between genotype and phenotype pattern.

Efflux pumps expel antibiotics, including β-lactams, aminoglycosides, tetracyclines and fluoroquinolones in Gram-negative bacteria, particularly in non-fermentative bacilli (Vila et al., 2007). The major facilitator superfamilies (MFS) and resistance-nodulation-division (RND) families are the most common efflux pumps in A. baumannii (Vila et al., 2007). The present study assessed adeB (RND), tetA and tetB (MFS) efflux pumps for the collected isolates. The TetA and TetB efflux pumps have played an important role in resistance to tetracyclines. In studies from other countries, Yan et al. (2010) reported prevalent genes of tetA (26.5%) and tetB (65.3%) in the isolates of three hospitals in China. Huys et al. (2005) collected samples from different European hospitals and found that 32% of strains carried the tetB gene and 42% the tetA gene. These studies indicate that the prevalence of the tetA gene at Ghaem Hospital is lower than in other Iranian cities and in Europe and China. The present findings suggest that tetB and other tetracycline-resistant determinants (tetH, tetM, tetX and adeB), instead of the tetA gene, are responsible for tetracycline resistance in all the clinical isolates tested. The correlation between AdeABC efflux pumps (adeB) and the antibiotics studied was not meaningful (data not shown); however, the contribution of outer-membrane proteins should not be neglected. More study is required to determine the details of
the accessory-resistant profile of the Ghaem Hospital collection.

Clustering of isolates by REP-PCR revealed a clonal distribution in circulating clones of different wards of Ghaem Hospital. Various resistance genes in this clone suggest a plasmid-borne transfer among isolates. Transformation and conjugation experiments are of value to address this issue.

It should be stressed that the expression level is important in some of the resistance genes studied. For example, coexistence of MBL genes like VIM with ISAba1/OXA23 was observed in some isolates and it should be determined which gene confers resistance to β-lactam antibiotics. Real-time PCR could provide the solution to this problem. One limitation of this study was the lack of ability to provide more discrimination among isolates. PFGE could overcome this problem; however, with existing equipment, REP-PCR was the only choice for determining the clonal relationship of isolates.

In conclusion, the findings reveal that the presence of OXA-23, OXA-24, blaVIM and ISAba1 could be responsible for carbapenem resistance in clinical isolates of A. baumannii. Moreover, tetracycline resistance is probably provided by the TetB efflux pump for all strains, although the TetA efflux pump-encoding gene was absent in the isolates studied. Analysis of the prevalence of AME-encoding genes in clinical isolates of A. baumannii showed that most strains were positive for aadA1 and aphA1. The results of the present study confirm that the increase in refractory A. baumannii isolates is alarmingly high. This highlights the need for strict infection control measures and for the hospital control infection team to exert strict measures for infection control. A frequent-surveillance system should be considered. Further study is required to determine other resistance determinants of A. baumannii in isolates from Ghaem Hospital.

ACKNOWLEDGEMENTS

The authors would thank laboratory staff of Ghaem hospital for their technical assistance. This work was funded by the Mashhad University of Medical Sciences.

REFERENCES


