Genotyping of carbapenem resistant *Acinetobacter baumannii* isolated from tracheal tube discharge of hospitalized patients in intensive care units, Ahvaz, Iran

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**ABSTRACT**

**Background and Objectives:** Carbapenem resistant *A. baumannii* is an emerging cause of nosocomial infections. The aims of this study were identification of the most prevalent of carbapenem resistant genes, molecular typing and antimicrobial evaluation of *A. baumannii* in intensive care units.

**Materials and Methods:** Two hundred and six *A. baumannii* were isolated from tracheal tube discharge of hospitalized patients at different intensive care units in Ahvaz, Iran. Antimicrobial susceptibility test was done on all isolates. Multiplex and singleplex PCR were performed for detection of *bla*OXA-23-like*, bla*OXA-24-like*, *bla*OXA-51-like*, *bla*VIM, *bla*IMP, *bla*SPM and *bla*NDM genes. Genetic relationship of all isolates was determined by REP-PCR method.

**Results:** Out of 206 examined isolates, 198 (96.1%) isolates were resistant to imipenem and meropenem. However 3.9% isolates were sensitive to these antibiotics. The *bla*OXA-23-like* and *bla*OXA-24-like* genes were detected in 85% and 8.7% of strains, respectively. No *bla*OXA-58-like*, *bla*IMP, *bla*VIM, *bla*SPM and *bla*NDM were detected. REP-PCR results showed that isolates were belonged to five genotypes: Genotype A was the most prevalent (P-value < 0.001): it was observed in 75 of 206 strains (36.4%). Genotype B, and C were found in 28.6% and 27.7%, respectively. The rate of other genotypes was as follows: D (2.4%), E (1%).

**Conclusion:** Based on the obtained results, the rate of carbapenem resistance was high among of *A. baumannii* which was isolated from intensive care units patients and oxacillinase genes were the most prevalent carbapenem resistant genes. These results revealed that three clones, A, B and C of *A. baumannii* are common in our hospitals.

**Keywords:** *Acinetobacter baumannii*, Carbapenem resistant, Intensive Care Units

**INTRODUCTION**

*Acinetobacter baumannii* has been recognized over the last decades as a major pathogen that responsible for severe infections, especially in the Intensive Care Units (ICU) patients (1, 2). Ventilator-associated pneumonia (VAP) occurs in critically ill patients who admitted to ICU and *A. baumannii* frequently isolated in respiratory specimens of these patients (2, 3). VAP complications are increased mortality rate, prolonged hospitalization and high medical costs (4, 5). Although, previously, most of *A. baumannii* isolates were sensitive to carbapenems, and imipenem was the gold standard treatment for *A. baumannii* pneumonia.
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(1, 5), widespread use of carbapenems has led to an emergence of resistant strains (6). Nowadays, carbapenem resistant A. baumannii (CRAB) has been reported worldwide, and it has become a global problem (7). In addition, an important concern is nosocomial infection causing by multidrug resistant A. baumannii (MDRAB) isolates (8). Due to increasing resistance, selection of an appropriate therapeutic option is difficult (9). The main mechanisms of carbapenem resistance in A. baumannii, is the production of carbapenem-hydrolyzing enzymes, mostly OXA types carbapenemases such as blaOXA-23-like, blaOXA-24-like, blaOXA-58-like and blaOXA-143-like, and less common by metallo-β-lactamas (7, 10). In order to control and prevention of spread of resistant isolates, it is necessary to use a molecular method for typing of MDRAB A. baumannii (8). Because no study has been performed independently solely on A. baumannii tracheal isolates in Ahvaz, this study was planned with the following objectives: (i) to study the antimicrobial susceptibility pattern (ii) to identify the most prevalent OXA types and metallo-β-lactamase genes and (iii) To characterize clonal relationship among A. baumannii isolates collected from the tracheal tube discharge in Ahvaz, south west of Iran using repetitive sequence-based polymerase chain reaction (REP-PCR).

MATERIALS AND METHODS

Bacterial isolates. This study was conducted at the 8 ICUs of 2 teaching hospitals, Imam Khomeyni and Golestan, associated to Ahvaz Jundishapur University of Medical Sciences from March 2010 to November 2012. A total of 206 A. baumannii isolates were collected from a tracheal tube discharge of ICUs patients which were colonized or infected by these bacteria. The patients were hospitalized in ICU sections such as: ICU internal, surgery, A & B, neurosurgical ICU (NSICU), general 1, general 2, cardiovascualar ICU (CVICU). Initial identifications of A. baumannii were performed using standard laboratory tests (11).

Antimicrobial susceptibility test. All isolates were subjected to antimicrobial susceptibility testing by standard disk diffusion method according to Clinical and Laboratory Standard Institute (CLSI) guidelines (12). The tested antimicrobial agents were as follows: imipenem 10 µg, meropenem 10 µg, polymyxin B 300 unit, gentamicin 10 µg, ceftriaxone 30 µg, colistin 10 µg, piperacillin 100 µg, piperacillin-tazobactam 100/10 µg, cefepime 30 µg tobramycin 10 µg, tigecyclin 15 µg, amikacin 30 µg, tetracycline 30 µg, ciprofloxacin 5 µg, trimethoprism-sulfamethoxazole 1.25/25.75 µg, ceftazidine 30 µg, rifampin 5 µg, aztreonam 30 µg and ampicillin-sulbactam (10/10 µg), (MAST, Group Ltd, Merseyside, UK). Reference strains include Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 were used for quality control testing (12). The results of disk diffusion were interpreted by CLSI. The US Food and Drug Administration-approved criteria for enterobacteriaceae used for tigecycline breakpoint (13).

Extraction of DNA. DNA was extracted by phenol–chloroform method with some modification. After growth of bacteria on Mueller-Hinton’s agar plates, 2 or 3 colonies were suspended in 180 µl of distilled water. Then, 20 µl of proteinase K (1 mg/ml) was added and after adding 200µl of buffer solution (0.5% SDS, 0.005 M EDTA and 0.01 M Tric-Cl, pH 7.8) mixture was incubated at 55°C for 2 hours. Then 200 µl of phenol and 200 µl of chloroform were added and gently mixed. After centrifugation at 11000 rpm for 5 minutes at 4°C, the supernatant was collected and transferred to a new tube carefully. To precipitate of DNA, 50µl of ammonium acetate 7.5 M and 200 µl of absolute ethanol were added. DNA was removed and transferred to a new tube. After washing three times with 70% ethanol, DNA was placed at 30°C overnight. Dried DNA was dissolved in 100µl of TE buffer (14). The DNA purity of extracted was measured by photobiometer (Eppendorf, Germany) in 260/280 nm UV long waves.

Detection of blaOXA-51-like. To confirm the identity of A. baumannii, blaOXA-51-like gene was sought by PCR assay using specific primers listed in Table 1 (15). To amplify this gene, each reaction was carried out in a final volume of 25 µl containing 1x PCR buffer, 1U Taq polymerase, 1.5 mM MgCl₂, 200 µM of dNTP, 10 pmol of each primer (Eurofins MWG Operon, Germany) and 1 µl of extracted DNA. Amplification conditions were programmed in Mastercycler Eppendorf (Eppendorf, Germany) as follows: Initial denaturation at 94°C for 3 min; 35 cycles of 94°C for 45 s, annealing 57°Cfor 45 s, extension 72°C for 1min and final extension 72°C for 5 min. PCR products
were separated on 1.5% agarose gel (CinnaGen, Iran) by electrophoresis, stained with ethidium bromide (CinnaGen, Iran) and then visualized under UV gel documentation system. A. baumannii NCTC 12156 (ATCC 19606) was used as positive control. A negative control was included in each PCR reaction, containing all components except the DNA template which was replaced by distilled water (15, 16).

**Multiplex PCR for detection of oxacillinase genes.** *bla*<sub>OXA-23-like</sub>, *bla*<sub>OXA-24-like</sub> and *bla*<sub>OXA-58-like</sub> were sought for all isolates by multiplex PCR according to protocol as described previously (15). Each PCR reaction was performed in a final volume of 25 µl with 1x PCR buffer, 1U Taq polymerase, 2 mM MgCl₂, 200 µM of dNTP (CinnaGen, Iran), 0.2 µM of each primer (TAG, Copenhagen A/S Denmark) and 1 µl of template DNA. PCR conditions were programmed in Mastercycler Eppendorf (Eppendorf, Germany) as follows: Initial denaturation at 94°C for 10 min; followed by 30 cycles at 94°C for 30 s, 53°C for 40 s and 72°C for 50 s and final extension at 72°C for 6 min. PCR products were separated with electrophoresis on 1.5% agarose gel (CinnaGen, Iran) and after staining with ethidium bromide, visualized under UV gel documentation system. *A. baumannii* reference strains including NCTC 13304, NCTC 13302, NCTC 13305 used as positive control for *bla*<sub>OXA-23-like</sub>, *bla*<sub>OXA-24-like</sub> and *bla*<sub>OXA-58-like</sub> respectively (15).

**Detection of metallo β-lactamase genes.** The presence of different metallo β-lactamase encoding genes such as: *bla*<sub>IMP</sub>, *bla*<sub>VIM</sub>, *bla*<sub>SPM</sub> and *bla*<sub>NDM</sub> were investigated by PCR with specific primers that were listed in (Table 1) and protocol from two previous studies (16, 17).

**REP-PCR.** In order to determine genetic relationship of carbapenem resistant *A. baumannii*, REP-PCR was carried out on all isolates with specific primers previously described (18) and are listed in (Table 1). Amplification condition was performed based on the method of Bou et al. with some modifications (14). Each reaction mixture was done in volume of 25 µl with 1x PCR buffer, 3.5 mM of MgCl₂, 300 µM of dNTP, 3% DMSO (Cinna Gen, Iran), 0.5 µM of each primer (TAG, Copenhagen A/S Denmark), and 1U of Taq polymerase and 1 µl of genomic DNA, PCR conditions were as follows: 94°C for 10 min; 30 cycles of 94°C for 1 min, annealing temperatures 45°C for 1 min and 72°C for 2 min and 72°C for 16

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**Table 1. Primers sequences used in this study.**

| Primer name | Sequence (5'-3') | Amplicon size (bp) | Reference |
|-------------|----------------|--------------------|-----------|
| bla<sub>OXA-51-like</sub>-F | TAATGCTTTGATCGGCCTTG | 353 | (16) |
| bla<sub>OXA-51-like</sub>-R | TGGATGCTCCTGCTCGGTTG | | |
| bla<sub>OXA-23-like</sub>-F | GATCGGATTGGAGAACCAGA | 501 | | |
| bla<sub>OXA-23-like</sub>-R | ATTTCTGACCGCATTTCCAT | | |
| bla<sub>OXA-24-like</sub>-F | GGTTAGTTGGCCCCCTTAAA | 249 | | |
| bla<sub>OXA-24-like</sub>-R | AGTTGACGCGAAAAAGGGGATT | | |
| bla<sub>OXA-58-like</sub>-F | AAGTATTTGGGCTTGTGCTG | 599 | | |
| bla<sub>OXA-58-like</sub>-R | CCCCTCCTGCGCTCTCACATA | | |
| bla<sub>IMP</sub>-F | TCGTTTGAAGGAATGTTAACGG | 568 | | |
| bla<sub>IMP</sub>-R | ATGTAAGTTCTCAAGAGTATGTC | | |
| bla<sub>VIM</sub>-F | GGTGGTGGTCGTCATCGGC | 502 | | |
| bla<sub>VIM</sub>-R | GTTCAGCCAGATCGCATCGGC | | |
| bla<sub>NDM</sub>-F | CCGAATGGCTCATCAGATC | 624 | | |
| bla<sub>NDM</sub>-R | AAAATCTGCGGCTAGAAACG | | |
| bla<sub>SPM</sub>-F | ACATTTATCGCGGTAACGG | 271 | | |
| bla<sub>SPM</sub>-R | CACATTATCGCGGTAACGG | | |
| REP-1 | IIIGCCCGGICATCAGGC | | |
| REP-2 | AGCTTCTATACGCGCTAC | | |

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min. Products were separated with electrophoresis on 1.2% agarose gel (Cinna Gen, Iran); after staining with ethidium bromide, they were visualized under UV gel documentation system; then they were photographed and compared together with visual inspection (14). All fingerprints were observed by one observer. Fingerprints were interpreted according to previous studies (18, 19).

Statistical analysis. The results were analyzed using the SPSS version 16 to obtain frequencies and comparison among clones. Non parametric Chi-square test was used ($X^2 = 156.6, df = 5$). A P-value $< 0.001$ was considered statistically significant.

RESULTS

Bacterial isolates and antimicrobial susceptibility pattern. The rates of isolation from different wards were as follows: ICU Internal (41.3%), Surgery (14.1%), B (13.1%), Neurosurgical Intensive Care Unit NICU (9.2%), A (7.8%), General 1 (5.3%), General 2 (7.8%), Cardiovascular Intensive Care Unit (CVICU) (1.5%). All 206 studied isolates contained blaOxa-51-like gene and were known as A. baumannii. All isolates (100%) were susceptible to colistin and polymyxin B. Maximum resistance rate was observed for aztreonam and rifampin with 98.5% and 98.1%, respectively. The rate of resistance to antimicrobial agents is shown in Table 2.

Detection of Oxacillinase and Metallo-β-lactamase. Results of multiplex PCR showed the blaOXA-23-like and blaOXA-24-like genes were detected in 85% and 8.7%, respectively (Fig. 1). Six isolates were carbapenem resistant but these isolates were negative for blaOXA-58-like, blaIMP, blaVIM, and blaNDM were detected. blaOXA-23-like, blaOXA-24-like, and blaOXA-58-like were not detected in carbapenem susceptible A. baumannii (CSAB) isolates.

REP-PCR. To determine the genetic relationship, all isolates were analyzed by REP-PCR. According to the REP-PCR results, carbapenem resistant isolates were grouped into five clones. The first prevalent genotype was named genotype A and was observed in 75 (36.4%) of 206 isolates ($P$-value $< 0.001$), thereafter 59 isolates (28.6%) and 57 isolates (27.7%).

Table 2. The results of antibiogram test for A. baumannii isolates.

| Antibiotic                      | Sensitive (%) | Intermediate (%) | Resistant (%) |
|--------------------------------|---------------|-----------------|--------------|
| Imipenem 10 µg                 | 8 (3.9)       | -               | 198 (96.1)   |
| Meropenem 10 µg                | 8 (3.9)       | -               | 198 (96.1)   |
| Ceftazidime 30 µg              | 7 (3.4)       | 1 (0.5)         | 198 (96.1)   |
| Cefepime 30 µg                 | 8 (3.9)       | -               | 198 (96.1)   |
| Ceftriaxone 30 µg              | 1 (0.5)       | 7 (3.4)         | 198 (96.1)   |
| Colistin 10 µg                 | 206 (100)     | -               | 0            |
| Piperacillin 100 µg            | 6 (2.9)       | 1 (0.5)         | 199 (96.6)   |
| Piperacillin-tazobactam 100/10 µg | 8 (3.9)       | 0               | 198 (96.1)   |
| Polymyxin B 300 unit           | 206 (100)     | 0               | 0            |
| Gentamicin 10 µg               | 33 (16)       | 2 (1)           | 171 (83)     |
| Tobramycin 10 µg               | 44 (21.4)     | 0               | 162 (78.6)   |
| Amikacin 30 µg                 | 13 (6.3)      | 10 (4.9)        | 183 (88.8)   |
| Tetracycline 30 µg             | 15 (7.3)      | 16 (7.8)        | 175 (85)     |
| Ampicillin-sulbactam 10/10 µg  | 34 (16.5)     | 29 (14.1)       | 143 (69.4)   |
| Ciprofloxacin 5 µg             | 7 (3.4)       | 0               | 199 (96.6)   |
| Trimethoprim-sulfamethoxazole 1.25/23.75 µg | 16 (7.8)     | 5 (2.4)         | 185 (89.8)   |
| Rifampin 5 µg                  | 0             | 4 (1.9)         | 202 (98.1)   |
| Aztreonam 30 µg                | 0             | 3 (1.5)         | 203 (98.5)   |
| Tigecycline 15 µg              | 13 (6.3)      | 66 (32)         | 127 (61.7)   |

All antibiotics and antimicrobial susceptibility pattern. The rates of isolation from different wards were as follows: ICU Internal (41.3%), Surgery (14.1%), B (13.1%), Neurosurgical Intensive Care Unit NICU (9.2%), A (7.8%), General 1 (5.3%), General 2 (7.8%), Cardiovascular Intensive Care Unit (CVICU) (1.5%). All 206 studied isolates contained blaOxa-51-like gene and were known as A. baumannii.

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belonged to genotypes B and C, respectively (Fig. 2). The rates of other genotypes were as follows: D: 5 (2.4%), E: 2 (1%). All clones were distributed throughout the study period. Albeit bla\textsubscript{OXA-23-like} was found in all clones, bla\textsubscript{OXA-24-like} was seen only in clones A, B, C and D, but clones E was negative for this gene. Eight isolates (3.9%) were carbapenem susceptible and showed different REP pattern from each other. Frequency of each clone in different ICUs was presented in Table 3.

**DISCUSSION**

*A. baumannii* is the third causative agent of infection at ICUs patients with mortality rates of 26-68% (20). Increasing rate of carbapenem resistant *A. baumannii* is an important concern (7). Production of Class D oxacillinase which is distributed worldwide is the main mechanism of resistance to carbapenems in this organism. The major carbapenemase genes involved in carbapenem resistance are *bla*\textsubscript{OXA-24-like}*, *bla*\textsubscript{OXA-23-like}, *bla*\textsubscript{OXA-51-like} respectively.

Table 3. Frequency of each clone of carbapenem resistant and carbapenem susceptible isolates in different ICUs.

| Clone | Internal | Surgery | A | B | NICU* | General 1 | General 2 | CVICUb | Total |
|-------|----------|---------|---|---|-------|-----------|-----------|--------|-------|
| A     | 36       | 7       | 5 | 9 | 6     | 1         | 9         | 2      | 75    |
| B     | 31       | 10      | 1 | 3 | 4     | 6         | 3         | 1      | 59    |
| C     | 11       | 8       | 9 | 14| 8     | 4         | 3         | 0      | 57    |
| D     | 2        | 2       | 0 | 0 | 0     | 0         | 1         | 0      | 5     |
| E     | 1        | 1       | 0 | 0 | 0     | 0         | 0         | 0      | 2     |
| Sensitive | 4     | 1       | 1 | 1 | 1     | 0         | 0         | 0      | 8     |
| Total | 85       | 29      | 16| 27| 19    | 11        | 16        | 3      | 206   |

*Neurosurgical ICU
Cardiovascular ICU
24). Despite some authors reported bla\textsubscript{OXA-51-like} from 2% to 84.92% in A. baumannii (8, 23, 25, 26) but we did not find any bla\textsubscript{OXA-51-like} gene in accordance with previous studies (6, 10, 24). Interestingly, we identified 6 isolates that were resistant to imipenem and meropenem that possessed only the intrinsic bla\textsubscript{OXA-51-like} but other investigated genes were negative. Resistance to carbapenems in these isolates may be in related to other mechanisms including decreased permeability, alteration of penicillin-binding proteins, AmpC stable derepression and overexpression of efflux pump (27). In A. baumannii, metallo-β-lactamase genes such as: bla\textsubscript{IMP}, bla\textsubscript{VIM}, bla\textsubscript{SPM}, and bla\textsubscript{NDM} have been reported sporadically (28-32) but we did not find any of these genes in our isolates. Similar to our results, negative finding for bla\textsubscript{IMP}, bla\textsubscript{VIM}, bla\textsubscript{SPM}, and bla\textsubscript{NDM} has been reported by some authors (6, 8, 24, 33). REP-PCR revealed three main genotypes are present in our hospitals and 92.7% of isolates belonged to three genotypes A, B and C. Nosocomial outbreaks of carbapenem resistant A. baumannii were reported worldwide and in most cases one or two epidemic isolates were involved (34) and certain clones are disseminated in the hospitals (8, 24, 35). Since, the majority of our isolates belonged to three main genotypes, it is possible that cross-transmission with similar organisms has been occurred between patients. Therefore, it seems that these clones are circulating in our hospitals. Yan et al. reported that multidrug resistant A. baumannii from same genotype can be transferred between hospitals and even cities (8). Albeit bla\textsubscript{Oxa-23-like} was found among all resistant clones, Interestingly only genotypes A, B, C and D were positive for bla\textsubscript{Oxa-23-like} and this gene was negative in genotypes E. Of note, each sensitive isolates had unique REP-PCR patterns that were different from carbapenem resistant isolates and it is assumed that probable source of this isolates were outside of the studied hospitals. However, it has been reported that carbapenem susceptible isolates may have highly similar or different REP pattern from the carbapenem resistant isolates (35). Our finding revealed that in addition to carbapenems, the rates of resistance to other antimicrobial agents were high. Dizbay et al. 2008 in Turkey studied on susceptibility pattern of 66 isolates of A. baumannii collected from endotracheal aspirate and found resistance rate to imipenem, and meropenem was 80.3% and 71.2%, respectively. Different rate of resistance to other tested antibiotics were reported by Dizbay et al. (9).

The rates of resistance to all antimicrobial agents in our study are higher than reported by Dizbay et al. In conclusion, REP-PCR revealed that three main genotypes involved in the two studied hospitals. In order to prevent the spread of resistant isolates between different wards and patients, we suggest it is necessary to monitor and improve infection-control procedures. Although the research was carefully performed and was reached its aims, but there was one limitation. In our study, difference between colonization and infection was not clear and it suggested that in future studies this subject be surveyed.

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