**DETECTION OF AMBLER CLASS A, B AND D β-LACTAMASES AMONG PSEUDOMONAS AERUGINOSA AND ACINETOBACTER BAUMANNII CLINICAL ISOLATES FROM BURN PATIENTS**

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**SUMMARY.** In this study, we evaluated the existence of classes A, B and D β-lactamases among Pseudomonas aeruginosa (P.aeruginosa) and Acinetobacter baumannii (A.baumannii) strains isolated from burn patients in Tehran during the years 2012 and 2013. From these strains, the frequency of MBL (metallo-beta-lactamase) and ESBL (extended-spectrum beta-lactamase) producers were evaluated using CDDT (Combined Disk Diffusion Tests). The prevalence of some related genes, including bla_imp, bla_vim, bla_esp, bla_kpc, bla_dim, bla_bic, blaCTX-M-15 and blaNDM genes, was evaluated using PCR and sequencing methods. Of the 75 non-fermenter isolates, 47 P.aeruginosa and 28 A.baumannii were isolated and identified. A high rate of resistance to common antibiotics was detected among A.baumannii isolates in particular, showing 100% resistance to 9 tested antibiotics. CDDT showed that 21 (28%) and 25 (34.25%) of the non-fermenter isolates were ESBL and MBL producers respectively. The prevalence of blaCTX-M-15 and blaNDM genes among the 75 non-fermenter isolates was 7 (9.3%) and 1 (1.3%), respectively. Fortunately, no other genes were detected in either of the non-fermenters. The mortality rate due to MBL-producing isolates was 5 (20%). This study showed specific resistance genes exist among some MBL and ESBL gram-negative non-fermenters which were isolated from burn patients in Tehran.

**Keywords:** Pseudomonas aeruginosa, Acinetobacter baumannii, ESBLs, burn patients, MBLs

**Introduction**

Burn patients are at risk of acquiring infection because of their damaged skin and broken immune system. Pseudomonas aeruginosa (P.aeruginosa) and Acinetobacter baumannii (A.baumannii) are common gram-negative non-fermenter opportunistic bacteria associated with nosocomial infections, especially in immune-compromised patients admitted to an intensive care unit (ICU). They are also a leading cause of morbidity and mortality among hospitalized burn patients. Nowadays, increasing rates of drug resistance among bacteria is a major concern worldwide. The most common mechanism of resistance is the production of β-lactamases, including enzymes of Ambler classes A, D and B, with the corresponding genes often associated with mobile genetic elements such as plasmids. Due to the wide ranging distribution of the metallo-β-lactamases (MBL, Class B), potent carbapenemase activity and resistance to inhibitors, these enzymes can confer resistance to almost all β-lactams. Since the 1990s, several metallo-β-lactamases encoded by mobile DNA have emerged in important pathogens (i.e., in Enterobacteriaceae, P.aeruginosa, and A.baumannii). Several genotypes of MBLs have been documented in P.aeruginosa and A.baumannii, including bla_imp, bla_vim, bla_esp, bla_kpc, bla_dim, bla_bic, blaCTX-M-15 and blaNDM genes. Extended-Spectrum-beta-Lactamases (ESBLs) are Ambler Class A β-lactamases, which can hydrolyse monobactams and cephalosporins but not carbapenems or cephamycins. They are inhibited by clavulanate. There are various genotypes of ESBLs such as bla_SHV, TEM, and blaCTX-M types. Other types include blaPER, VEB, PER, BEL-1, BES-1, STII, PRO, and VEB. The OXA-type β-lactamases (class D) are often detected in P.aeruginosa but also in many other gram-negative bacteria such as A.baumannii. They were initially characterized by their high rates of hydrolysis of oxacillin and cloxacillin antibiotics. Therefore, the aim of this study was to determine the frequency of bla_dim, bla_esp, bla_kpc, bla_bic, bla_CTX-M-15 and blaNDM type genes among P.aeruginosa and A.baumannii isolates from hospitalized burn patients in the Shahid Motahari Hospital, Tehran, Iran during the years 2012 and 2013.

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Materials and methods

Bacterial identification
From April to July 2012, 75 non-fermenter gram-negative bacilli were isolated from 240 wound samples of burn patients admitted to the Burn Unit of Shahid Motahari Hospital (Tehran, Iran). The wound exudates were collected by swabbing and immediately transported to the microbiology laboratory of the Department of Microbiology of Shahid Beheshti University of Medical Sciences, Tehran, Iran. Isolation and identification was done using standard methods\(^9\) and confirmed by the Microgen identification kit (Microgen\(^\text{TM}\), UK).

Antimicrobial susceptibility testing
The Kirby-Bauer disk diffusion method on Mueller Hinton agar (Merck, Germany), based on Clinical Laboratory Standards Institute (CLSI) guidelines 2012,\(^8\) was used to perform antimicrobial susceptibility tests on imipenem (IPM: 10µg), meropenem (MEM: 10µg), ceftazidime (CAZ: 30µg), cefotaxime (CTX: 30µg), amikacin (AK: 30µg), tobramycin (TOB: 10µg), piperacillin/tazobactam (PTZ: 100/10µg), ciprofloxacin (CIP: 5µg), cefepoxide (CDP: 10µg), aztreonam (ATM: 30µg) and gentamicin (GEN: 10µg), all purchased from Mast Group, Merseyside, UK. P.aeruginosa ATCC27853 and A.baumannii ATCC19606 were used as control strains.

Minimum Inhibitory Concentration (MIC)
Strains found resistant to IPM and CAZ by disk diffusion test were re-checked in broth microdilution method according to the guidelines of the CLSI 2012.\(^7\) MIC for imipenem (Jaberebne Hayyan Co) and CAZ (GLAXO England Co.) ranging from 0.5 µg/ml to 256 µg/ml was tested. Also, P.aeruginosa ATCC 27853 was used as the control strain.

Phenotypic detection of MBL
CDDT was performed for identification of MBLs by IPM and MEM alone and in combination with EDTA. The inhibition zones of the IPM and IPM+EDTA, MEM and MEM+EDTA were compared after 18 hours of incubation at 37°C. A zone diameter difference between the discs alone and the discs+EDTA ≥7mm was interpreted as a positive result for MBL production.\(^1\)

Phenotypic detection of ESBL
Detection of ESBLs was tested for all the isolates by CDDT containing CAZ and CTX alone, with CAZ 30µg+clavulanic (CA: 10µg) and CTX 30µg+CA 10µg per disc. The zone of inhibitions were compared for the CTX and CAZ discs alone to the CAZ 30µg+CA 10µg the and CTX 30µg+CA 10µg disc. An increase in zone diameter of ≥5mm in the presence of clavulanic acid indicated the presence of ESBL in the test organism. Escherichia coli ATCC 25922 and Klebsiella pneumoniae ATCC700603 were used respectively as negative and positive controls for ESBL production.\(^7\)

DNA extraction
Total DNAs of the different bacterial isolates were extracted by alkaline lysis method as described before.\(^10\)

PCR and Sequencing methods
PCR method was used for screening of the bla\(_{\text{IMP}}\), bla\(_{\text{VIM}}\), bla\(_{\text{SPM}}\), bla\(_{\text{KPC}}\), bla\(_{\text{GIM}}\), bla\(_{\text{DIM}}\), bla\(_{\text{BIC}}\), bla\(_{\text{OXA-48}}\) and bla\(_{\text{NDM}}\) genes. The primers and PCR programs used in this study were as previously described.\(^6\) The predictive PCR product sizes were: 232 bp for (IMP-F and IMP-R), 390 bp for (VIM-F and R), 271 bp for (SPM-F and R), 477 bp for (GIM-F and R), 438 bp for (OXA-48-F and R), 537 bp for (BIC-F and R),621 bp for (NDM-F and R), 699 bp for (DIM-F and R), and 798 bp for (KPC-F and R), respectively.\(^11\) Amplification for bla\(_{\text{CTX-M-15}}\) gene was performed with primers CTX-M-15-F (5’-GCATGGGCAGTACCAGTAA-3′) and CTX-M-15-R (5’-TTACCCAGCGTCAGATTCCG -3′). Amplification was carried out with the following thermal cycling conditions: 5 min at 94°C and 36 cycles of amplification consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with 5 min at 72°C for the final extension. PCR product bands were analysed after electrophoresis in a 1% agarose gel at 95 V for 45 min in 1X TBE containing Ethidium Bromide under UV irradiation. The PCR purification kit (Bioneer Co., Korea) was used to purify PCR products and sequencing was performed by the Bioneer Company (Korea). The nucleotide sequences were analyzed with Chromas 1.45 software and BLAST in NCBI.

Statistical Analysis
This study was descriptive-application. For analysis of results, MINITAB16 software was used. P.value and confidence intervals were <0.05 and 95%, respectively.

Results
In total, 75 non-fermenter gram-negative bacilli were isolated from 240 wound samples from burn patients admitted to the Burn Unit at Shahid Motahari Hospital (Tehran, Iran). Of the 75 non-fermenter isolates, 47 (62.67%) were identified as P.aeruginosa and 28 (36.33%) as A.baumannii. The antimicrobial resistance data are presented in Table I. The MICs of CAZ and IMP for P.aeruginosa and A.baumannii are presented in Table II.

The CDDT results showed that 15 (31.91%) of the P.aeruginosa isolates and 6 (21.42%) of the A.baumannii were positive for production of ESBLs. It also revealed...
that 13 (17.8%) of the *P. aeruginosa* isolates and 12 (16.4%) of the *A. baumannii* isolates were positive for production of MBLs. The frequency of ESBL and MBL genes among *P. aeruginosa* and *A. baumannii*, which were detected by PCR method, are shown in Table III.

The mortality rate due to metallo-lactamases-producing *P. aeruginosa* and *A. baumannii* infection was 5 (20%) among the hospitalized burn patients.

### Discussion

Isolation of *P. aeruginosa* and *A. baumannii* strains from burn centers is frequent and has been reported by various global studies, including from Iran. The frequency of ESBL and MBL genes among *P. aeruginosa* and *A. baumannii*, which were detected by PCR method, are shown in Table III.

The mortality rate due to metallo-lactamases-producing *P. aeruginosa* and *A. baumannii* infection was 5 (20%) among the hospitalized burn patients.

### Table I - Antibacterial resistance among tested *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th><em>Pseudomonas aeruginosa</em> Resistant No (%)</th>
<th><em>Acinetobacter baumannii</em> Resistant No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem (IMP: 10μg)</td>
<td>78.73</td>
<td>100</td>
</tr>
<tr>
<td>Meropenem (MEM: 10μg)</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>Aztreonam (ATM: 10μg)</td>
<td>82.98</td>
<td>100</td>
</tr>
<tr>
<td>Gentamicin (GEN: 10μg)</td>
<td>72.72</td>
<td>96.85</td>
</tr>
<tr>
<td>Ciprofloxacin (CIP: 30μg)</td>
<td>69.23</td>
<td>100</td>
</tr>
<tr>
<td>Ceftazidime (CAZ: 30μg)</td>
<td>72.72</td>
<td>100</td>
</tr>
<tr>
<td>Cefotaxime (CTX: 30μg)</td>
<td>82.98</td>
<td>100</td>
</tr>
<tr>
<td>Cefepime (FEP: 30μg)</td>
<td>67.57</td>
<td>100</td>
</tr>
<tr>
<td>Ceftriaxone (CRO: 30μg)</td>
<td>82.98</td>
<td>100</td>
</tr>
</tbody>
</table>

### Table II - Minimum Inhibitory Concentration (MIC) of ceftazidime and imipenem among *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>Acinetobacter baumannii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC50 (µg/mL)</td>
<td>MIC90 (µg/mL)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>128</td>
<td>256</td>
</tr>
</tbody>
</table>

### Table III - Results for β-lactamase genes in 47 *Pseudomonas aeruginosa* isolates and 28 *Acinetobacter baumannii* isolates by PCR and Sequencing methods

<table>
<thead>
<tr>
<th>Organisms</th>
<th>No. (%) of positive isolates for β-lactamases genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>bla&lt;sub&gt;IMP&lt;/sub&gt;</td>
</tr>
<tr>
<td>Forty-seven <em>Pseudomonas aeruginosa</em> isolates</td>
<td>1 (2.1%)</td>
</tr>
<tr>
<td>Twenty-eight <em>Acinetobacter baumannii</em> isolates</td>
<td>0</td>
</tr>
</tbody>
</table>
tibiotics are presently the only restricted antibiotics which are recommended for treating burn patients in Iran. Infection type is a major determinant of the initial regimen. Traditional antibiograms provide information on the susceptibility of bacteria to a range of drugs, but they do not answer the question of what antibiotic combinations may be optimal against any given bacteria. Combination antibiotic therapy is important as an initial antibiotic therapy for infections in which *A. baumannii* and *P. aeruginosa* are prominent. Carbapenems with another active agent in vitro (colistin or an aminoglycoside) were associated with distinctly lower mortality rates than were carbapenems alone.

A further importance of these bacteria is related to the different resistance mechanisms with which they are armed. Several global studies state that their resistance to beta-lactams is related to various enzymes that are produced, including extended-spectrum beta-lactamases (ESBLs) and metallo-beta-lactamas (MBLs) which belong to Ambler classes A and B. In this study, using CDDT method, 47 of the *P. aeruginosa* isolates (62.67%) and 28 of the *A. baumannii* isolates (36.33%) were identified as MBL producers. Most of the time, the MBL producers can hydrolyse a wide range of antibiotics, with the exception of aztreonam. However, in this study, most *P. aeruginosa* isolates (82.98%) and all *A. baumannii* strains (100%) were aztreonam resistant in parallel to their MBL producing ability. From these results we can conclude that we must change the old definition for A and B beta-lactamase by Ambler or modify the phenotypic methods.

Usually, restriction in phenotypic methods urge researchers to confirm phenotypic results by molecular methods, although there are different genes which are coded beta-lactamases. Among MBL genes, IMP is more important, especially in Iran, albeit first reported from Japan in 1980. The other gene is bla*_{vmp}\textsubscript{\text{\textsuperscript{1}}}*, which was previously reported from Ahwaz, another city in Iran. In our study, the IMP enzyme was identified in only one *P. aeruginosa* strain by PCR and further sequencing, but was not detected among any of the isolated *A. baumannii* strains. Also, the bla*_{vmp}\textsubscript{\text{\textsuperscript{1}}}* gene was not detected among either *P. aeruginosa* strains or *A. baumannii* strains. These results are in contrast to those of two other studies from Iran: in Tehran 11.43% of *P. aeruginosa* strains and in Ahwaz 19.51% *P. aeruginosa* isolates were reported to have the VIM gene. Also, Owlia et al. reported that 94% of *P. aeruginosa* isolates from Tehran were identified as MBL producers and carried the bla*_{vmp}\textsubscript{\text{\textsuperscript{1}}}* gene. While in 2012, Fallah et al. showed that, similar to our study, *P. aeruginosa* isolates from burn patients only had the bla*_{vmp}\textsubscript{\text{\textsuperscript{1}}}* gene. Even so, these data are in contrast to ours and, although the reason for this discrepancy is not completely clear, this may be related to differences in the time of the studies and consequently to changes in antibiotics prescriptions or the primers used. Also, other studies from Asia, such as the ones from Malaysia, detected bla*_{\text{Vmp}}* and bla*_{\text{IMP}}* from *P. aeruginosa* and *A. baumannii* isolates respectively, or bla*_{\text{IMP}}* gene which was detected from *P. aeruginosa* isolates. In addition, this kind of discrepancy was seen among some studies from other Middle Eastern countries, such as a study from Saudi Arabia which detected VIM, another study from Lebanon which reported IMP-1, and a study from Oman which detected NDM-1.

The other MBL coding gene is bla*_{\text{OXA-41}}* which was identified recently and first reported from New Delhi, India, followed by other countries including Pakistan. Due to the close proximity of these countries to Iran and the facility of bidirectional trips between them, as well as the ease of transfer of resistance among these kinds of bacteria, we predicted a high likelihood of detecting this gene among our isolated *P. aeruginosa* and *A. baumannii* strains. We hoped not to detect this gene among the non-fermenter bacteria which were isolated from Tehran in this study. Even so, we continued to screen for this gene among other *P. aeruginosa* and *A. baumannii* strains isolated from different cities which border or are close to Pakistan. These kinds of studies are valuable for preventing the distribution of resistant bacteria to other parts of the world.

The other class D beta-lactamases encoding gene is bla*_{\text{OXA-48}}* which was first reported from Turkey. Clearly Turkey is another of Iran’s neighbours and any existence of this gene among Iranian isolated *P. aeruginosa* or *A. baumannii* strains would not be unexpected. Fortunately, this gene was not detected among our tested bacteria. However, in order to confirm that this gene does not exist in Iran, further studies may be required with larger sample sizes and from a wider range of cities, especially those close to Turkey.

In the case of the bla*_{\text{SMP-1}}* gene which is responsible for MBL production, SPM-1 was detected in only 1 out of the 13 MBL positive *A. baumannii* strains (1.3%) using PCR. This means that this gene does not exist in any of the *P. aeruginosa* strains isolated in our study. However, in 2010, Shahcheraghi et al. reported 6 out of 100 *A. baumannii* isolates to be MBL producers, and this gene was detected in all 6 strains. Although at first glance our results appear dissimilar, deeper study shows low frequency of this gene among Tehran isolated *A. baumannii* strains. Furthermore, there are no reports on the frequency of this gene among Iranian *P. aeruginosa* isolates. However, in Brazil, six *A. baumannii* strains which were resistant to penicillins, cephalosporins and carbapenems had bla*_{\text{SMP-1}}*.

In contrast to the expectation of other beta-lactamase genes like bla*_{\text{NDM-1}}* and bla*_{\text{KPC}}* from European countries or bla*_{\text{PC}}* from the USA, fortunately we did not identify any of these among our *P. aeruginosa* and *A. baumannii* isolates.

Based on the existing data, *P. aeruginosa* and *A. baumannii* are growing to become the most important common
ESBL producing bacteria, consequently making their eradication difficult.

In this study, 31.91% of *P. aeruginosa* isolates were ESBL-positive, which is in accordance with other studies conducted in Iran (28%), Bangladesh (37.8%) and India (32.6%). However, it is in contrast with two other studies from Iran whose results were lower (18%) and higher (42.8%) than ours. This discrepancy may be related to the increased usage of beta-lactam drugs nowadays and to the difference in the time of study. In addition, 21.42% of *A. baumannii* were identified as ESBL producers through phenotypic tests, which was similar to the findings of Owlia et al. (21%) in Iran and another study in Poland (20%).

The high rate of ESBL prevalence in Iran and its widespread dissemination is a cause for concern. In this study, the existence of blaCTX-M-15 was detected in 4 (30.7%) of the *P. aeruginosa* and 3 (25%) of the MBL-producing *A. baumannii* isolates, respectively. This is of particular concern in Iran where the ESBL prevalence is very high.

In this study, we also carefully monitored the mortality rate of burn patients related to isolated gram-negative non-fermenter MBL producers. This subject is particularly important as regards more severe infections and reports of mortality rates ranging from 25 to 75%, which are related to these carbapenem resistant *P. aeruginosa* and *A. baumannii* strains in comparison to other IMP resistant bacteria. Unfortunately, 5 (20%) of the mortality cases of burn patients in this study were related to MBL producer strains. However, this rate is low in comparison to that found in India - which was 50% related to *P. aeruginosa* isolates and 44.4% by *A. baumannii* strains - or in comparison to the 82.6% mortality rate from *P. aeruginosa* in Brazil.

Through accurate screening of ESBL and MBL enzymes and further precise supervision of hospital practitioners, it will be possible to control the spread of multidrug resistant non-fermenter strains and decrease the associated cases of fatality, especially in burn patients.

**Conclusion**

The prevalence of beta-lactamase-producing isolates and their isolation from life-threatening infections such as those found in burn patients is increasing at an alarming rate worldwide. Increased pressure for usage of antimicrobial drugs in the treatment of burns has resulted in the eradication of normal flora, which may be a cause of the substitution of MDR isolates. It was shown in this study that beta-lactamase producing *P. aeruginosa* and *A. baumannii* strains are an emerging threat in burn care units and should be supervised by implementation of timely identification and strict isolation methods that will help to reduce their severe outcomes and mortality rates in these patients.

Finally, clinicians should apply logic in selection and use of drugs in treating severely ill patients. Data is available with which to make a more logical choice of drug, and closer liaison with a microbiologist will greatly facilitate the decision-making process.

**RÉSUMÉ.** Dans cette étude, nous avons évalué l’existence des classes A, B et D de β-lactamas chez les souches de *Pseudomonas aeruginosa* (*P. aeruginosa*) et *Acinetobacter baumannii* (*A. baumannii*) isolées des patients brûlés à Téhéran pendant les années 2012 et 2013. La fréquence des producteurs du MBL (metallo-bêta-lactamase) et du BLSE (bêta-lactamase à spectre étendu) s’est élevée par les tests de diffusion sur les disques combinés, et la prévalence de certains gènes liés (y compris les gènes *blaIMP*, *blaCTX-M-15*, *blaVIM*, *blaSPM*, *blaKPC*, *blaGIM*, *blaDIM*, *blaBIC*, *blaOXA-48*, *blaCTX-M-15* et *blaNDM*) a été vérifiée avec des tests pour réaction en chaine à la polymérase et les méthodes de séquençage. Un total de 75 isolats non-fermentateurs ont été isolés et identifiés : 47 étaient *P. aeruginosa* et 28 étaient *A. baumannii*. Un taux élevé de résistance aux antibiotiques communs a été détecté parmi les isolats d’*A. baumannii* en particulier, avec une résistance de 100% aux 9 antibiotiques testés. Les tests de diffusion des disques combinés a montré que 21 (28%) et 25 (34,25 %) des isolats non-fermentateurs étaient producteurs du BLSE et du MBL, respectivement. La prévalence des gènes *blaCTX-M-15* et *blaIMP* parmi les 75 isolats non-fermenteurs était 7 (9,3%) et 1 (1,3%), respectivement. Heureusement, d’autres gènes n’ont pas été détectés. Le taux de mortalité due aux isolats producteurs des MBL était 5 (20%). Cette étude a montré que des gènes avec des résistances spécifiques existent parmi certains non-fermenteurs MBL et BLSE Gram-négatifs qui ont été isolés des patients brûlés à Téhéran.

**Mots-clés:** *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, BLSE, patients brûlés, MBL

**BIBLIOGRAPHY**


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