Investigation of Antimicrobial Resistant Patterns and Prevalence of Carbapenamase Genes (imp-1, vim-2, and kpc) in MDR Pseudomonas aeruginosa Strains Isolated from Patients in Mottahari Hospital in Tehran, Iran

A B S T R A C T

Aims The aim of this study was to identify antibiotic resistant patterns and the prevalence rate of carbapenem resistant genes (imp-1, vim-2, kpc) in P. aeruginosa strains isolated from burn patients in Shahid Motahari Hospital of Tehran.

Materials & Methods In this study, 63 P. aeruginosa strains were collected from infected patients. Isolates were identified by biochemical tests and specific 16SrDNA PCR. Antibiotic susceptibility test was performed by standard Kirby-Bauer method according to the CLSI guidelines. The prevalence of imp-1, vim-2, and kpc genes were assessed by PCR.

Findings All of the isolates were confirmed as P. aeruginosa by phenotypic tests and specific 16SrDNA PCR. Totally, 14 antibiotypes were identified. The highest resistance was observed against tobramycin, gentamicin, amoxi-clavulanic acid, and ceftoxitin (100%) and the most sensitivity was shown against colistin (100%). All of the isolates were multidrug resistant (MDR), 100 and 46% were positive for Extended Spectrum β-Lactamases (ESBL) and Metallo-β-Lactamases (MBLs) respectively. The imp-1 and kpc genes were not detected (0%), while vim-2 gene was present in all of the isolates.

Conclusion In the current study, the high resistance rate to antibiotics might be due to their overuse for burn patients as a prophylactic or therapeutic agents. Colistin is considered a drug of choice for the treatment of wounds infected by P. aeruginosa in burn patients. In this study, the majority of P. aeruginosa isolates belonged to Antibiotype 1 and possess carbapenemase vim-2. Therefore, to stop this resistance transmission, the prevention and control are apparently essential.

Keywords P. aeruginosa; Burns; KPC; VIM; IMP

C I T A T I O N  L I N K S

Introduction

*Pseudomonas aeruginosa* is an aerobic gram-negative and non-fermentative bacterium and one of the most etiological agents of nosocomial and opportunistic infections in lungs, urinary tract, surgical site, and sepsis [1, 2]. This bacterium is an important pathogen in burn patients, so that multidrug resistant (MDR) *P. aeruginosa* is a major pathogen, which is present in the moist parts of wounds and leads to mortality [2-3]. This organism is characterized by innate and acquired resistance to different classes of antimicrobial agents, including beta-lactams (penicillin, cephalosporins, monobactams, carbapenems), aminoglycosides, fluoroquinolones, and lipopeptides [4]. The mechanisms involved in *P. aeruginosa* resistance to beta-lactam antibiotics include genetic mutations, transmission of lactamase genes, increase in the expression of efflux pump genes, and reduced penetration to cell membrane [5].

According to Ambler classification (1980), the beta-lactamases is comprised of 4 classes: A- Extended Spectrum Beta-Lactamases (ESBL), B- Metallo- beta-Lactamases (MBL), C- Cephalosporinases (AmpC), and D- Oxacillinas (OXA) [6].

MBLs are one of the most important families of beta-lactamases, mainly regarding their transmission by mobile genetic elements. Instead of serine in the active site of beta-lactamases, MBLs carry Zn^{2+}. Serine enzymes eliminate amide bonds in the beta-lactam ring to inactivate the antibiotics. MBLs also catalyze the same chemical reaction using Zn^{2+}[6].

The acquired MBLs include VIM, IMP, SPM-1, GIM-1, SIM-1, AIM-1, KHM-1, NDM-1, and DIM-1. Generally, *imp* and *vim* type enzymes, which are encoded by integron, contain several types [7, 8]. The carbapenemases are an important group of beta-lactamases deactivating carbapenems, which are one of the most effective beta-lactams against MDR gram-positive and gram-negative bacteria such as *P. aeruginosa* [6].

Most types of transferable carbapenemases have been detected in *P. aeruginosa* isolates worldwide, among which MBLs are of great clinical significance [9]. KPC (*Klebsiella pneumoniae* carbapenemase), which belongs to Ambler Class A carbapenemase and its presence in *P. aeruginosa* isolates was first reported from Colombia [10], creates mighty resistance due to high rates of carbapenem hydrolysis; consequently, there is no need for other mechanisms like efflux pumps or impermeability. The Class C and D beta-lactamases have rarely been found in *P. aeruginosa*, therefore, do not have the same clinical importance [10-12].

Detecting the presence of carbapenemase activity in pathogenic bacteria is a critical issue for infection control because it is often associated with extensive resistance to different classes of antibiotics, treatment inefficiency, and mortality.

Objectives: Therefore, the aim of this study was to investigate and determine the antibiotic resistant pattern or antibiotyp: type of isolates based on resistant patterns against different classes of antibiotics and prevalence of carbapenem resistant genes including *imp-1, vim-2, and kpc* in MDR *P. aeruginosa* strains isolated from burn patients in Mottahari Hospital in Tehran.

Materials and Methods

Bacterial isolates: In this study, 63 *P. aeruginosa* isolates were collected from wound, blood, and catheter of infected patients hospitalized in Mottahari Hospital in Tehran from June to October, 2016. The demographic information of patients including gender, age, and unit of hospitalization were collected. The isolates were identified by biochemical tests, including Gram staining, citrate, catalase, oxidase, the growth on MacConkey agar, Triple sugar Iron agar (TSI), oxidative-fermentative test (OF), growth at 42°C, and Methyl Red Voges Proskauer (MRVP). All culture media were purchased from Merck company distributor (Merck; Germany). The PCR assay was performed by 16s rRNA specific primer pairs of *P. aeruginosa* to confirm the identification. The genomic DNA of isolates was extracted by boiling and subjected to PCR assay for confirmation.

Antimicrobial susceptibility testing: Antibiotic susceptibility test was performed by Kirby-Bauer disc diffusion method on Mueller-Hinton Agar (MHA; Merck; Germany) according to the Clinical Laboratory Standard Institute (CLSI) guidelines [13-14]. The antibiotic disks (Padtan teb, IRAN) used in this research include ciprofloxacin (5 μg), amikacin (30 μg), gentamicin (10 μg), tobramycin (10 μg), colistin (10 μg), ceftazidime (30 μg), ceftazidime/clavulanic acid (30/10 μg), aztreonam (30 μg), amoxi-clavulanic acid (30 μg), imipenem (10 μg), cefotaxime (30 μg), cefotaxime/clavulanic acid (30/10 μg), cefoxitin (30 μg), ticarcillin (75 μg), piperacillin (100 μg), and piperacillin/tazobactam (100/10 μg).

The suspension of bacteria (0.5 McFarland) was prepared and streaked on MHA (Merck; Germany) by sterile swabs, and, then, antibiotic discs were placed on with 20 mm distance from each other. Then, the plates were incubated at 37°C for 18-24 hours.

The double-disk synergy test was used for the detection of MBLs production. In this assay, two IPM disks were used, one of which contains EDTA (0.5M), the inhibition zone more than 7 mm was considered as positive. Ethylene- Diamine- Tetra-Acetic acid (EDTA) as a poly amino carboxylic can act as a chelating agent and attach to metal ions such as Zn^{2+} and disable them [15, 16].

The Extended Spectrum Beta-Lactamase (ESBL) producing isolates were detected by the double disc...
synergy test (DDST), using ceftazidime (30 μg) and cefotaxime discs (30 μg) with and without clavulanic acid (10 μg). The inhibition regions difference 5 mm between discs with and without clavulanic acid was considered as ESBL producer [17]. MDR isolates were determined by WHONET 2017 software (O’Brien and Stelling Co).

**PCR amplification assay:** DNA extraction was performed by boiling method. One loopful of overnight grown bacteria on Brain Heart Infusion (BHI) agar plates (Merck; Germany) was picked up and mixed in 200 μL sterile deionized water and boiled for 10 min. After centrifugation in 12000 rpm, the supernatant was subjected for PCR by *imp-1*, *vim-2*, and *kpc* primers; their sequences are presented in Table 1.

### Table 1) Primers used in this study

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Primer sequence (5′ to 3′)</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imp_1</td>
<td>F : CTACCGCAGCATGGCTTTTG</td>
<td>587bp</td>
<td>[43]</td>
</tr>
<tr>
<td></td>
<td>R : AAACGATTTGGCCATTTGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vim-2</td>
<td>F : ATGGCTGTTTGTGCATATC</td>
<td>510bp</td>
<td>[44]</td>
</tr>
<tr>
<td></td>
<td>R : TGGGCCATTTCCGAGATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>kpc</td>
<td>F : AGTTCCTTGGTTTCATTC</td>
<td>798bp</td>
<td>[45]</td>
</tr>
<tr>
<td></td>
<td>R : CTGTGCTTGTCATCTGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16srRNA</td>
<td>F : GGAGATCTTGGAGACATCA</td>
<td>956bp</td>
<td>[46]</td>
</tr>
<tr>
<td></td>
<td>R : TCCTTAGAGTGCCGACCG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The amplification assay was performed, using a sensquest LabCycler (Germany) in a volume of 12.5 μL containing 1 μL of template DNA (500 ng.μL⁻¹), PCR Master-mix (6.5 μL; Ampliqon; Denmark), and 0.5 μL of each primer (10 pmol).

The PCR conditions were as follow: an initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 20s; annealing at 57, 59, and 58°C regarding *imp*, *vim* and *kpc*, respectively for 20s; and an extension at 72°C for 40s, followed by a final extension at 72°C for 2 min. PCR products were run on electrophoresis by 1.5 and 1% agarose gel for *imp*-1 and *vim*-2, and for *kpc*, respectively, and visualized by UV documentation (Uvitec Cambridge; France).

*Acinetobacter baumannii* AC54/97 [18], *P. aeruginosa* COL-1 [19], and *K. pneumoniae* A08053 [20] were used as positive control for *imp*, *vim*, and *kpc* genes, respectively.

**Statistical analysis:** Pearson’s Chi-square test was performed to evaluate the correlation between antibiotic resistance and presence of carbapenemase gene, MBLs, and, ESBLs production. A significant level of 0.05 was considered for this test.

### Table 2) Antibiotic resistant and susceptibility

<table>
<thead>
<tr>
<th>Antibiotic Disk</th>
<th>Resistant (%)</th>
<th>Susceptibility (%)</th>
<th>Intermediate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamycin (10μg)</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tobramycin (10μg)</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amoxi-clavulanic acid (30μg)</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cefoxitin (30μg)</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Imipenem (10μg)</td>
<td>98.5</td>
<td>0</td>
<td>1.5</td>
</tr>
<tr>
<td>Ciprofloxacin (5μg)</td>
<td>98.5</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>Amikacin (30μg)</td>
<td>98.5</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>Ticarcillin (75μg)</td>
<td>92.5</td>
<td>0</td>
<td>7.5</td>
</tr>
<tr>
<td>Pipercillin-tazobactam (100/100μg)</td>
<td>90.5</td>
<td>1.5</td>
<td>8</td>
</tr>
<tr>
<td>Pipercillin (100μg)</td>
<td>87.5</td>
<td>0</td>
<td>12.5</td>
</tr>
<tr>
<td>Ceftazidime (30μg)</td>
<td>76.1</td>
<td>23.9</td>
<td>0</td>
</tr>
<tr>
<td>Azteronam (30μg)</td>
<td>65.5</td>
<td>3</td>
<td>31.7</td>
</tr>
<tr>
<td>Colistin (10μg)</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

**Findings**

**Bacterial isolate:** Totally, 63 isolates were obtained from burn patients with wound infection in Motahhari Hospital of Tehran from June to October, 2016. These isolates were collected from wound (84.1%), blood (6.3%), catheters (6.3%), and tissue (1.5%). 79% of the infected patients were men and 21% were women. The patients aged between 1-81 years old and 90% were between 20-55 years old. 73, 16, and 11% were hospitalized in men, women, and pediatric units, respectively. All isolates (100%) were identified and confirmed as *P. aeruginosa* through microbiologic and biochemical standard tests and PCR analysis for 16srRNA.

**Antimicrobial susceptibility testing:** The antibiotic resistance and antibiotyping pattern of *P. aeruginosa* isolates are displayed in Table 2 and 3, respectively. As shown in Table 2, the most resistance was observed against gentamycin (100%), tobramycin (100%), amoxi-clavulanic acid (100%), cefoxitin (100%), ciprofloxacin (98.5%), amikacin (98.5%), and imipenem (98.5%), whereas the most sensitivity was observed to colistin (100%) and ceftazidime (23.9%; Table 2).
As shown in Table 3, 14 antibiotypes were determined, in which 52.3% of the isolates belonged to Pattern 1. All of the isolates (100%) were positive in double-disk synergy assay for MBL production (Figure 1A). Overall, 29 isolates (46%) were positive in double disc synergy assay for ESBL production (Figure 1B). MDR observed in all isolates was determined by WHONET application software (2017). The frequency comparison of the two groups (antibiotic resistant and ESBLs or MBLs production) was displayed (p<0.05).

**PCR amplification assay:** Totally, among 63 isolated samples, 63 isolates (100%) were confirmed as *P. aeruginosa* by 16srDNA PCR (Figure 2 A). The *imp-1* and *kpc* genes were detected in none of the isolates, whereas *vim-2* was positive in all of the isolates (100%; Figure 2B-D). The Pearson’s Chi-square analysis showed the comparison of the frequency of the two groups (the resistance of isolates to carbapenems and the presence of *vim-2*) is statistically significant (p<0.05).

**Table 3** Antibiotyping of isolates

<table>
<thead>
<tr>
<th>Antibiotypes</th>
<th>Antibiogram Pattern</th>
<th>Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotype 1</td>
<td>R R R R R R R R S R</td>
<td>52.3</td>
</tr>
<tr>
<td>Antibiotype 2</td>
<td>R R R R I R R R S R</td>
<td>12.6</td>
</tr>
<tr>
<td>Antibiotype 3</td>
<td>R R S I R R R R S R</td>
<td>9.5</td>
</tr>
<tr>
<td>Antibiotype 4</td>
<td>R R S R R R R S R R</td>
<td>7.9</td>
</tr>
<tr>
<td>Antibiotype 5</td>
<td>I R S R R R R S R R</td>
<td>3.1</td>
</tr>
<tr>
<td>Antibiotype 6</td>
<td>I R R R R R R R R S</td>
<td>1.5</td>
</tr>
<tr>
<td>Antibiotype 7</td>
<td>I S S I R R R R S R</td>
<td>1.5</td>
</tr>
<tr>
<td>Antibiotype 8</td>
<td>R R R R I R R R S S</td>
<td>1.5</td>
</tr>
<tr>
<td>Antibiotype 9</td>
<td>I I S I R R R R S S</td>
<td>1.5</td>
</tr>
<tr>
<td>Antibiotype 10</td>
<td>I R R I R R R R S I</td>
<td>1.5</td>
</tr>
<tr>
<td>Antibiotype 11</td>
<td>I I R I R R R R S I</td>
<td>1.5</td>
</tr>
<tr>
<td>Antibiotype 12</td>
<td>I I R S R R R R S I</td>
<td>1.5</td>
</tr>
<tr>
<td>Antibiotype 13</td>
<td>R I S S R R R R S I</td>
<td>1.5</td>
</tr>
<tr>
<td>Antibiotype 14</td>
<td>R R R I R R R R S I</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Figure 1** A and B represent the producing isolates of MBLa and ESBL, respectively

**Figure 2** A: PCR amplification of 16srDNA F-R, Lanes N, P representatives of negative and positive controls, Lanes 1-6 *P. aeruginosa* isolates, M: 100bp DNA size marker. 2B-D: PCR amplification of *kpc*, *vim-2*, and *imp-1* genes, respectively with positive and negative controls
Discussion

Resistance to a wide variety of antimicrobial agents is an increasing global health threat, limiting drug choice, and making it difficult to control and treat infections [21]. MDR P. aeruginosa is an important agent in nosocomial infections. This bacterium is one of the most important microorganisms creating several clinical problem as a result of high antibiotic resistance [22].

In this study, 79% of the infected patients were men and 21% were women; given the high risk of men activities, the results seem obvious. The patients aged between 1-81 years old and 90% were between 20-55 years old, which is the age of employment and more probably work with flammable and dangerous equipment. Totally, 73, 16, and 11% were hospitalized in men, women, and pediatric units, respectively.

In present study, the antibiotic sensitivity and prevalence of carbapenem resistant genes (imp-1, vim-2, and kpc) were determined in P. aeruginosa strains isolated from burn patients of Motahari Burn Center in Tehran, Iran in 2016. All of the strains were shown to possess MDR features based on the definition explaining, whose isolate is resistant to at least 3 groups of antibiotics. Totally, 14 antibiotic types were identified in the current study, and more than half of the isolates belonged to Antibiotype 1 (52.3%) with the highest resistance level (Table 3). This Antibiotype has been distributed between strains isolated from men, women with different age, and different hospital units. We assume, it is due to the high rate resistant of this type, which affects its circulation in different units and patients with different gender and age range. As was shown in Table 3, the frequency of different isolates decreases from Antibiotype 1 to Antibiotype 14, and it may relate to sensitize the isolates to some antibiotics.

Approximately, all of the isolates were resistant to gentamycin, cefotaxin, tobramycin, imipenem, ciprofloxacin, and amikacin, whereas 23.9, 34.5, and 100% of the isolates were sensitive to ceftazidime, aztreonam, and colistin, respectively (Table 2). The MBLs activity was detected in all of the isolates (100%), whereas 29 isolates (46 %) were positive in double disk synergy test for ESBLs. The production of the enzymes statistically correlates with antibiotic resistant (p<0.05). In this cross sectional study, the high antibiotic resistance level in isolates might be due to the overuse of different groups of antibiotics for the prophylaxis and treatment of burn patients.

In a study performed by Golshani et al. in Isfahan hospitals, resistance to antibiotics including gentamycin, amikacin, ceftazidime, tobramycin, and imipenem was reported to be 60, 70, 68, 62, and 58%, respectively [23]. In another study performed by Fazeli et al. in Isfahan university teaching hospital in Iran, resistance to ceftazidime was 71%, and 11 isolates (31.4%) were positive for ESBL production, 45% of which were resistant to imipenem, and 51% were resistant to meropenem, 9 of which were MBL producers [8]. The lower rate of antibiotic resistance in the mentioned studies compared to the present study may be related to the extent of antibiotic usage and resistance in burn center during 3 years.

In another study carried out by Ghanbarzadeh et al., the resistance was reported to ciprofloxacin (93.7%), amikacin (82%), aztreonam (86.8%), piperacillin (85.4%), ceftazidime (82.6%), and imipenem (79.2%); in addition, 93.1% of the isolates were MDR [4]. The results their study are approximately similar to susceptibility assay results of our study.

In the present study, all of the isolates (100%) were susceptible to colistin, consistent with Akhi et al.’s study. It seems that colistin can be considered as a responsive therapiotic agent [24].

There are several studies concluding that P. aeruginosa isolates and gram-negative bacilli resistant to carbapenems are increasing rapidly in Asia, Europe, and South America [25-27]. The results of the present study showed that all of the P. aeruginosa isolates (100%) lack kpc gene; this result is similar to other studies reported from Iran, indicating low prevalence of kpc gene [28, 29].

In a study by Pakhbaten Toukpanlou et al. conducted on infected burn injuries in Tehran, most of the isolates (88%) were MDR, and none of the 50 imipenem resistant isolates possessed kpc gene [30]. Their study and the present study revealed that resistance rate is increasing, and fortunately, kpc gene has not yet been transmitted to studied isolates [30].

The diversity in the obtained results may be due to different used methods, samples, and variation in bacterial species.

Our results demonstrated that whereas 46% of isolates produced ESBLs, P. aeruginosa has the highest ESBL production, followed by other gram-negative bacilli such as A. baumannii [31-33]. In addition to intrinsic resistance to cephalosporins and aztreonam, ESBL-producing organisms have shown that co-resistance to many other classes of antibiotics like quinolones and aminoglycosides is mainly due to restriction in therapeutic options. The most remarkable risk factors for infection with ESBL-producing organisms in burn patients include long-term antibiotic usage, lengthy hospitalization, and overuse of third-generation cephalosporin and invasive methods [34, 35]. Even though β-lactamase inhibitors possess significant activity against ESBL in vitro, their clinical effects against serious infections caused by ESBL-producing organisms is polemical [36, 37].

In the present study, the phenotypic tests indicated that all of the isolate were MBLs producer, and PCR test indicated that the presence of vim gene was
positive in all of the isolates, while \textit{imp-1} gene was present in none of the isolates. This finding is in accordance with the phenotypic tests. Correspondingly, it seems that the high resistance to carbapenem (imipenem 98.5\%) in this study is due to the production of VIM. Of course, other mechanisms are involved in carbapenem resistance, highlighting the need for more investigation.

The production of MBL enzymes such as \textit{vim} and \textit{imp} is one of the most important reasons for resistance against carbapenems, which is highly prevalent in \textit{P. aeruginosa}. Several studies have indicated that carbapenems resistance in \textit{P. aeruginosa} isolates has been risen \cite{38-40} that is consistent with our results.

In a study performed by Abiri \textit{et al.} in Kermanshah University of Medical Science, of 76 imipenem resistant \textit{P. aeruginosa} isolates, 34 (75\%) isolates carried \textit{imp-1} gene, and 1 (2.2\%) isolate carried \textit{vim-2} gene \cite{41}. In another study by Aghamiri \textit{et al.}, of 100 imipenem resistant \textit{P. aeruginosa} isolates collected from 9 different hospitals in Tehran, 70 isolates carried \textit{vim} gene, and 20 isolates had \textit{imp} gene \cite{42}. The low prevalence of \textit{imp} gene compared to \textit{vim-2} in the mentioned study is comparable with this study results.

Comparing with the results of other studies, the current study exhibited an obvious rise of antibiotic resistance in \textit{P. aeruginosa} isolates in Iran. This study indicated that most of the MBLs positive \textit{P. aeruginosa} isolates carried \textit{\beta}-lactamase genes, and horizontal and vertical transfer between the bacterial species, strains, and integrons leads to the development of antibiotic resistance in the global level. Therefore, the focus on antibiotics resistant patterns either phenotypic or genotypic properties is crucial.

Conclusion

In this study, the high resistance rate against to antibiotics might be due to the excessive use of antibiotics for prophylaxis and treatment of burn patients. Hence, appropriate use of antibiotics is necessary. Colistin is a proper option for the treatment of wounds infected by \textit{P. aeruginosa} in burn patients. The majority of \textit{P. aeruginosa} isolates under study belonged to Antibotype 1, and the prevention and control of this resistance transmission is apparently essential.

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Conflict of Interests: The authors declare no conflict of interest.

Authors’ Contribution: Arash Abednezhad (First author), Introduction author/ Original researcher/ (35\%); Pourya Nasimoghadas (Second author), Assistant/ Statistical analyst (35\%); Nastaran Asghari Moghaddam (Third author), Methodologist/ Discussion author (30\%).

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