

Detection of antibiotic resistance genes among *Pseudomonas aeruginosa* strains isolated from Burn Patients in Iran

Abstract

Aim: In this study, we evaluated the presence of antibiotic resistance genes among *P.aeruginosa* strains.

Methodology: From January to September 2012, 100 isolates of *P.aeruginosa* were collected from burn patients. Antimicrobial susceptibility testing was performed by disk diffusion method. Screening for Metallo- β -lactamases (MBLs) productions were performed by Combination Disk Diffusion Test (CDDT). The frequency of antibiotic resistance encoding genes such as MBLs (IMP, VIM, NDM), ESBLs (CTX-M-15), Amp-C enzyme (CMY), Ambler class A carbapenemases (KPC), Ambler class D β -lactamase (OXA-48) , 16S rRNA methylases (armA, rmtB, rmtC , rmtD), Quinolone Resistance Gene (aac(6')-Ib) and presence of class 1 integron were detected.

Results: 48(62.33%) of isolates were metallo-beta-lactamase producers. All MBL-producing *P. aeruginosa* were resistant to antibiotics; while 49% of isolates were resistant to Gentamicin. The aac(6)-Ib, CTX-M-15, *int* I, CMY, rmtB , rmtD and IMP-1 genes were detected in 57 (74.02%), 48 (62.3%), 48 (62.3%), 7 (9.09%), 11 (14.28%), 9 (11.68%) and 6 (7.7%) isolates respectively, whereas none of them were positive for other genes. The mortality rate due to metallo- β -lactamases-producing *P. aeruginosa* infection was 5(10.4%).

Conclusions: The prevalence of antibiotic resistance genes producing *P. aeruginosa* detected in this study is of great concern.

23 **Keywords:** *Pseudomonas aeruginosa*, Burnt patients, β -lactamases, Antibiotic Resistance
24 Genes

25 1. Introduction

26 Burn injuries are among the most common and devastating forms of trauma. According to
27 The National Centre for Injury Prevention and Control in the United States, around two
28 million fires occur each year which leave 1.2 million people injured with burns and cause
29 About 5,000 deaths annually from burn-related complications. Thermal destruction of the
30 skin as a protective barrier and consequent depression of host immune responses are
31 important factors contributing to infectious complications in patients with severe burn injury
32 (1). Following thermal injury, burn wound surfaces are sterile immediately; these wounds
33 eventually become colonized with microorganisms though. The nature and degree of the
34 thermal damage along with the types and amounts of microorganisms colonizing the burn
35 wound appear to influence the future risk of an invasive wound infection. Although
36 *Staphylococcus aureus* remains a common cause of early burn wound infection,
37 *Pseudomonas aeruginosa* from the patient's endogenous gastrointestinal flora and/or an
38 environmental source is the most common cause of burn wound infections in many centers.
39 *P. aeruginosa* can cause a wide range of infections such as septicemia, pneumonia,
40 endocarditis, urinary tract infection, skin, ears and eyes infections in patients with impaired
41 immune system (2,3). Carbapenems are the most potent antibacterial agents for the treatment
42 of *P. aeruginosa* infections. However, carbapenem resistance due to metallo- β -lactamase
43 (MBL, Class B) production of *P. aeruginosa* has been reported (4). Several families of
44 MBLs have been classified in *P. aeruginosa*, including Imipenemase (IMP), São Paulo
45 metallo- β -lactamases (SPM), Verona integron-encoded metallo- β -lactamases (VIM), Seoul
46 imipenemase (SIM), Japan, Kyorin University Hospital Imipenemase (KHM), German
47 imipenemase (GIM) , New-Delhi metallo-beta-lactamase-1 (NDM-1) and Australian

48 *Imipenemase* (AIM) (5). Most β -lactamase genes are located on integrons which often
 49 encode clinically important drug resistance genes, such as extended-spectrum β -lactamases
 50 (ESBLs), MBLs and also 16S rRNA Methylase. Extended-Spectrum-beta-Lactamases
 51 (ESBLs) are Ambler Class A β -lactamases which can hydrolyse monobactams and
 52 cephalosporins but not carbapenems or cephamycins. There are various genotypes of ESBLs
 53 such as SHV, TEM, and CTX-M types. Other clinically types include KPC, VEB, PER, BEL-
 54 1, BES-1, SFO-1, TLA, and BIC (6). Also, the production of 16S rRNA methylase has been
 55 reported to be a novel mechanism of aminoglycoside resistance. So far, seven types of
 56 methylases have been detected (ArmA, RmtC, RmtA, RmtE, RmtB, RmtD and NpmA) (7).
 57 The aim of the present study was to determine the frequency of antibiotic resistance genes
 58 among *P. aeruginosa* isolates from hospitalized burn patients admitted to Shahid Motahari
 59 Hospital, Tehran-Iran during 2012 and 2013 years.

60

61 **2. Materials and methods**

62 **Bacterial identification**

63

64 From January to September 2012, 100 strains of *P. aeruginosa* were isolated from 448 burn
 65 patients admitted to Shahid Motahari Hospital. Samples were collected using sterile swab and
 66 had been transported in Stuart media to the laboratory, Cultured on Cetrimide and
 67 MacConkey agar then incubated at 37°C for 24 h. Colonies with pigment or grape-like odor,
 68 were further studied using biochemical tests such as oxidase test, catalase, sugar fermentation
 69 test and the ability of growth in 42°C. The isolates were stored at -20°C in brain heart broth
 70 containing 20% glycerol. *Pseudomonas aeruginosa* ATCC27853 was used as a control strain
 71 (7).

72 Antimicrobial susceptibility testing

73 Antimicrobial susceptibility to ceftazidime (CAZ: 30µg), cefotaxime (CTX: 30µg), amikacin
74 (AK: 30µg), tobramycin (TOB: 10µg), piperacillin/Tazobactam (PTZ: 100/10 µg),
75 ciprofloxacin (CIP: 5 µg), Carbenicillin (CAR: 30µg), imipenem (IPM: 10µg), cefepime
76 (FEP: 30 µg), ceftriaxone (CRO: 30µg), meropenem (MEM: 10µg), aztreonam (ATM: 30µg)
77 and gentamicin (GEN: 10µg)(Mast Group, Merseyside, UK) was performed by the Kirby-
78 Bauer disk diffusion method on Mueller Hinton agar (Merck, Germany) as recommended by
79 the Clinical Laboratory Standards Institute (CLSI) guidelines 2012(8). *Pseudomonas*
80 *aeruginosa* ATCC27853 was used as the control strain for susceptibility testing.

81 Phenotypic detection of MBLs

82 Combination Disk Diffusion Test (CDDT) was performed for the identification of MBLs by
83 meropenem and imipenem (Mast Group, Merseyside, UK) alone and in combination with 10
84 µl of 0.5 M EDTA (Sigma). The inhibition zones of the meropenem and meropenem+EDTA,
85 imipenem and imipenem+EDTA were compared after 24 h of incubation at 37°C. An
86 increase of 7mm or more in zone diameter in the presence of EDTA compared to those with
87 IMP or MER tested alone was considered to be a positive test for the presence of an MBL(2).

88 PCR and Sequencing methods

89 The boiling method was used to prepare the DNA templates of the following genes: IMP, VIM,
90 CMY, aac(6')-Ib, *armA*, *rmtB*, *rmtC*, *rmtD*, KPC, NDM and OXA-48. The genes were
91 detected by PCR and Sequencing using described primers. (2, 7, 9). Amplification for CTX-M-15
92 and *int* genes was performed by the primers CTX-M-15-F (5'-
93 GCGATGGGCAGTACCAGTAA-3') and CTX-M-15-R (5'-
94 TTACCCAGCGTCAGATTCCG -3') and *Int1*-F (5'-GGTGTGGCGGGCTTCGTG-3') and
95 *Int1*-R (5'-GCATCCTCGGTTTTCTGG-3'), respectively. Amplification was carried out with

the following thermal cycling order: 5 min at 94°C and 36 cycles of amplification consisting of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C, with 5 min at 72°C for the final extension. PCR product bands were analysed by electrophoresis in a 1.5% agarose gel at 95 V for 45 min in TBE 1X containing ethidium bromide under UV irradiation. The Sequencing method was performed by Bioneer Company (Korea) and then sequences were analyzed with Chromas 1.45 software and BLAST at NCBI.

Statistical analysis:

The analysis of the results was performed using MINITAB 16 software. *P value* and confidence intervals were <0.05 and 95%, respectively.

3. Results

From January to September 2012, 100 strains of *P. aeruginosa* were isolated from 448 burn patients admitted to Shahid Motahari Hospital. Twenty-eight strains were isolated from female patients (22 %) and Seventy-two from males (72 %). The CDDT results showed that among 77 Imipenem resistant *P. aeruginosa* strains, 48 (62.33%) were metallo-beta-lactamase producers. All MBL-producing *P. aeruginosa* isolates were resistant to Meropenem, Imipenem, Ceftazidime, Cefotaxime, Amikacin, Tobramycin, Ciprofloxacin, Aztreonam, Piperacillin/Tazobactam, Ceftriaxone, Cefepime and Carbenicillin; while 49% of isolates were resistant to Gentamycin (Table 1). The *aac*(6)-Ib, CTX-M-15, *int* I, CMY, *rmt*B, *rmt*D and IMP-1 genes were detected in 57 (74.02%), 48 (62.3%), 48 (62.3%), 7 (9.09%), 11 (14.28%), 9 (11.68%) and 6 (7.7%) of the isolates respectively (2, 5). The VIM, KPC, NDM, OXA-48, *arm*A and *rmt*C genes were not detected. The nucleotide sequence data reported in this paper have been submitted to the GenBank sequence database and assigned accession no. JX648311 and JX644173.

4. Discussion

Multidrug-resistant and extensively-drug-resistant *P.aeruginosa* are increasing therapeutic challenges worldwide. Over the last decade, the increase of carbapenem resistance among *P.aeruginosa* isolates has been mostly due to impermeability because of OprD loss and the production of metallo- β -lactamases (MBL) (10). The CDDT results showed that among 77 Imipenem resistant *P. aeruginosa* isolates, 48 (62.33%) were metallo-beta-lactamase producers. In this study, all MBL-producing *P.aeruginosa* were resistant to all antibiotics, while 49% of the isolates were resistant to Gentamycin. The results of the present study showed that all isolates were resistant to aztreonam, which is known to be stable against the hydrolytic activity of MBLs (4). Shahcheraghi et al. assessed 350 *P. aeruginosa* clinical isolates collected from two Iranian general hospitals (Imam Khomeini Hospital and Tehran Children Center) and showed that only 5% of the isolates were resistant to imipenem. So the difference between our results and Shahcheraghi's results may be because of the difference between the antibiotic therapy regimens in our respective hospitals. In our research, a higher prevalence of MBLs was found in Tehran comparing figures reported from Spain, USA, India and Korea, which can be due to hospitalization time, care conditions and advised antibiotics. Among MBL genes IMP, which was first reported from Japan at 1980, is more prevalent especially in Iran. The other MBL gene is bla_{VIM}, which was reported before from Ahwaz another city of Iran(2). Doosti et al. in Zanjan province in Iran showed that 23 (56%) of *P. aeruginosa* carried VIM and 10 (24.3%) possessed IMP gene. Also, 31 (70.5%) isolates contained class 1 integron gene (11). Nikokar et al. in Iran showed that out of the 86 *P. aeruginosa* strains isolated from burn wound samples, 37 (43%) had class 1 integrons (12). Class 1 Integron containing bla_{VIM-2} gene in *P.aeruginosa* clinical strains was reported from Japan. It was demonstrated by several previous reports that the genes of both IMP- and VIM-type MBL are often encoded on mobile gene cassettes inserted into class 1 integrons. Most of

145 MBL-producing isolates 48 (62.3%) carried class 1 integron gene, which can easily spread
146 the resistance encoding genes among these isolates. Several studies have also reported
147 different frequencies of MBL positive isolates carrying class 1 integrons. The other MBL
148 coding gene is bla_{NDM-1} which was identified recently and reported from New Delhi / India
149 for the first time and after that from other countries including Pakistan. The close distance of
150 these countries to Iran and large number of trips between the countries on one side and the
151 ease of resistance transfer among bacteria on the other hand led us to think that it may be
152 probable for our isolates to have the same gene, but hopefully we could not detect this gene
153 among the isolated *P.aeruginosa* strains (10). However, we had continued to screen other
154 *P.aeruginosa* strains which were isolated from border cities close to Pakistan to detect
155 bla_{NDM-1}. These kinds of studies are valuable to prevent the distribution of resistant bacteria to
156 other parts of the world. A previous study showed that β -lactamase gene like, bla_{DIM} is
157 prevalent among European countries or bla_{KPC} in the USA, fortunately we could not detect
158 any of them among our *P.aeruginosa* isolates (13,14). Production of 16S rRNA methylases
159 confers a very high level of aminoglycoside resistance to the producers. In Asia, armA and
160 rmtB have been found among bacterial isolates in Japan, Korea, Taiwan and China (15). In
161 the present study, rmtB gene (14.28%), was more prevalent than the other 16S rRNA
162 methylases genes in *P.aeruginosa* isolates. Poirel et al. showed that one *E. coli* isolate
163 expressed CTX-M-15, together with ArmA and RmtB genes, conferring a high level of
164 resistance to aminoglycosides (16). High-level aminoglycoside resistance in human
165 pathogens conferred by 16S rRNAmethylases is of great concern in Iran. If ESBL and 16S
166 rRNAmethylase-positive bacterial isolates disseminate widely, the high level of pan-
167 aminoglycoside and cephalosporins resistance will certainly have a great impact on death
168 rates, and costs of care. In this study, we also carefully monitored the mortality rate of burn
169 patients infected with *P.aeruginosa* MBL producers (17,18, 19) .48 (62.33%) of the patients

were infected with MBL-producing *P. aeruginosa* strains and of whom 5 (10.4%) died. Only a few antibacterial drugs were operative against MBL-producing *P.aeruginosa* that were isolated from Motahari hospital. Consequently, control and treatment of these infections caused by the mentioned bacteria is difficult, therefore there is an urgent need for revised treatment protocols to prevent resistance genes spread among clinical isolates (20).

5. Conclusion

The prevalence of β -lactamase-producing isolates, and their isolation from life-threatening infections, is increasing worldwide considerably. Intensity pressure for usage of antimicrobial drugs by patients with burn infections, results in eradication of normal flora and consequent substitution of MDR strains. We have shown that β -lactamase producing *P.aeruginosa* are widespread in burn care units and should be supervised by working on timely identification and strict isolation methods that will help to reduce the mortality and morbidity rate in these patients.

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249 Table 1. Antimicrobial Susceptibility Test Results of *P. aeruginosa* isolates

Antibiotic	Resistant No. (%)	Intermediate No. (%)	Sensitive No. (%)
Aztreonam (10 µg)	48(100%)	0(0%)	0(0%)
Meropenem (10 µg)	48(100%)	0(0%)	0(0%)
Gentamicin(10 µg)	23(49%)	0(0%)	25(51%)
Ciprofloxacin (30 µg)	48(100%)	0(0%)	0(0%)
Amikacin (30 µg)	48(100%)	0(0%)	0(0%)
Ceftazidime (30 µg)	83(100%)	0(0%)	0(0%)
Imipenem(10 µg)	83(100%)	0(0%)	0(0%)
tobramycin	48(100%)	0(0%)	0(0%)
Carbenicillin	48(100%)	0(0%)	0(0%)
cefotaxime	48(100%)	0(0%)	0(0%)