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Major Article

Phenotypic and molecular detection of metallo-β-lactamase-producing *Pseudomonas aeruginosa* isolates from patients with burns in Tehran, Iran

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Abstract

Introduction: Health care-associated infections caused by metallo-β-lactamase (MBL)-producing *Pseudomonas aeruginosa* are a significant growing concern in patients with burns worldwide. The aims of this study were to determine the antibiotic susceptibility of and detect the presence of MBLs among *P. aeruginosa* isolates and assess their clonal relationship using enterobacterial repetitive intergenic consensus (ERIC)-PCR. **Methods:** Non-duplicated clinical isolates (160) of *P. aeruginosa* were collected from patients with burns at the Motahari Hospital in Tehran, Iran. All isolates were identified using standard laboratory methods and further characterized for antimicrobial susceptibility. Any carbapenem-resistant isolates were then examined for MBL production by the E-test and MBL-encoding genes were detected by PCR. The clonal relatedness of MBL-producing isolates was assessed by ERIC-PCR. **Results:** For multidrug-resistant isolates, the highest rates of susceptibility were observed for colistin 160 (100%), polymyxin B 160 (100%), and ceftazidime 32 (20%). In total, 69 (43.7%) isolates were identified as MBL producers. Twenty-eight (17.5%) isolates were positive for the $bla_{\text{VIM-1}}$ gene followed by the $bla_{\text{IMP-1}}$ (15.6%) and $bla_{\text{SPM-1}}$ (5.6%) genes. ERIC-PCR revealed three separate genotypes, where type A (76.8%) was the most prevalent, followed by B (20.3%), and then C (2.9%). **Conclusions:** Our present study found that the $bla_{\text{IMP-1}}$ and $bla_{\text{VIM-1}}$ genes were present at a significant frequency and also detected the $bla_{\text{SPM-1}}$ gene in *P. aeruginosa* isolates for the first time, highlighting the need for establishing suitable infection control measures to successfully treat patients and prevent further spread of these resistant organisms among patients with burns.

Keywords: *Pseudomonas aeruginosa.* Metallo-β-lactamases. Enterobacterial repetitive intergenic consensus-PCR.

INTRODUCTION

Burn infections are a major health concern due to increasing morbidity and mortality worldwide, especially within developing countries[1]. *Pseudomonas aeruginosa* is regarded as one of the most important opportunistic pathogens among aerobic non-fermentative bacterial species and causes nosocomial infections in patients with burns[2]. The appearance of resistance to routinely used antimicrobial agents among pathogens associated with burn wounds restricts the accessible therapeutic options available to effectively treat these infections, which is frequently associated with a high mortality rate[3]. Carbapenems have been effective in the treatment of severe infections caused by *P. aeruginosa*. However, resistance to carbapenems has been reported to now be on the rise throughout the world[4,5] and several carbapenem-hydrolyzing β-lactamases have been identified in *P. aeruginosa*. The most common mechanism of resistance to carbapenems involves the acquisition of carbapenem-hydrolyzing β-lactamases belonging to Ambler classes B (metalloenzymes) and D (oxacillinase)[6]. Metallo-β-lactamases (MBLs) can hydrolyze a wide variety of β-lactams, including penicillins, cephalosporins, and carbapenems[7]. Various types of MBL genes have been identified in *P. aeruginosa*, including the imipenemase (IMP), Verona integron-encoded metallo-β-lactamase (VIM), São Paulo metalo-β-lactamase (SPM), Germany imipenemase (GIM), Seoul imipenemase (SIM), and New Delhi metallo-β-lactamase (NDM) types. IMP and VIM are the most prevalent types of acquired MBLs[8,9]. IMP-type enzymes initially emerged in early 1991 in Japan, while the VIM type was initially reported in Italy. However, both types have been increasingly reported in other parts of the world[10]. SPM-1, i.e., Sao Paulo MBL, was initially identified in a clinical isolate of *P. aeruginosa* in Brazil in 1997, while the New Delhi MBL-1 (NDM-1) type of MBL was found in *Klebsiella pneumoniae* isolated from a Swedish patient with a previous history of hospitalization in India[8,11]. The genes encoding these enzymes are usually carried on integrins with some located on transferable plasmids that have the potential to horizontally spread among different bacterial

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species[12]. Several non-molecular techniques have been used to study these zinc-dependent enzymes with the application of chelating agents, such as ethylenediaminetetraacetic acid (EDTA), to inhibit the enzymatic activity[13]. There are no standard phenotypic confirmatory tests for the detection of MBLs among *P. aeruginosa* isolates[14]. Molecular methods are necessary to confirm the presence of MBL genes in clinical isolates and are also considered proper tools for screening. Polymerase chain reaction (PCR) detection has high sensitivity and reliability for detection of MBL genes among MBL-producing *P. aeruginosa* isolates[15].

The Motahari Burn Hospital is located in Tehran and is the most frequently referred burn center, accepting the majority of referrals from a number of medical centers in Iran. Determining the prevalence of MBLs in *P. aeruginosa* isolates from patients with burns is of particular importance because the appearance of resistance in such clinical isolates is usually coupled with the emergence of higher rates of treatment failure and mortality. At present, little is known about the molecular epidemiology of MBL-producing *P. aeruginosa* isolates in the Motahari Burn Hospital. Therefore, this current study aimed to characterize the antibiotic susceptibility of and detect the presence of $bla_{\text{IMP-1}}$, $bla_{\text{IMP-2,}}$ $bla_{\text{VIM-1}}$, $bla_{\text{VIM-2}}$, $bla_{\text{SPM-1}}$, bla_{GIM}, bla_{SIM}, and $bla_{\text{NDM-1}}$ genes among *P. aeruginosa* isolates and assess the clonal relationship of these isolates using enterobacterial repetitive intergenic consensus (ERIC)-PCR.

METHODS

Study design and bacterial isolates

This was a cross-sectional study in which a total of 160 non-duplicated *P. aeruginosa* isolates were collected from patients with burns referred to the Motahari Hospital in Tehran, Iran during the 15 months from March 2015 to June 2016. Bacterial species were identified using standard laboratory methods, including Gram staining, oxidase, motility, catalase, citrate, and oxidation-fermentation tests, and assessments of growth at 37 and 44°C[16]. Species identity was confirmed by detection of *oprI* and *oprL* genes as described previously[17]. The isolates were stored at -70°C in trypticase soy broth containing 20% glycerol and subcultured twice prior to testing.

Ethical considerations

This study was approved by the Ethics Committee of Qazvin University of Medical Sciences (code IR.QUMS.REC.1394.726). Written informed consent was obtained from all individuals enrolled in this study.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined using the Kirby-Bauer standard disk diffusion method on Mueller-Hinton agar plates (Merck Co., Germany) according to the Clinical and Laboratory Standards Institute guideline[18]. The antimicrobial agents used in our study (Mast Diagnostics Group Ltd, Merseyside, UK) were imipenem (10µg), meropenem (10µg), amikacin (30µg), ciprofloxacin (5µg), gentamicin (10µg), ceftazidime (30µg), cefepime (30µg), aztreonam (30µg), amoxicillin clavulanate (30µg), cefoxitin (30µg), piperacillin/tazobactam (100/10µg), gatifloxacin (5µg), colistin (10µg), and polymyxin B (300 units). The *P. aeruginosa* American Type Culture Collection 27853 strain was used as the control strain in antimicrobial susceptibility testing.

MBL E-test

The isolates that were fully or intermediately resistant to imipenem and/or meropenem were screened for MBL production using the MBL E-test (AB Biodisk, Solna, Sweden). MBL E-test strips were double-sided and contained a seven-dilution range of imipenem (4-256 µg/ml on one side and 1-64 µg/ml on the other) in the presence of a constant concentration of EDTA. A reduction in the imipenem maximum inhibitory concentration by ≥ 3 $\log_2$ dilutions in the presence of EDTA or a maximum inhibitory concentration ratio of imipenem/imipenem plus EDTA of ≥ 8 was considered MBL positive[19].

Detection of MBL-encoding genes

Carbapenem-resistant isolates were tested by PCR for MBL-encoding genes, including $bla_{\text{IMP-1}}$, $bla_{\text{IMP-2}}$, $bla_{\text{VIM-1}}$, $bla_{\text{VIM-2}}$, $bla_{\text{SPM-1}}$, bla_{GIM}, bla_{SIM}, and $bla_{\text{NDM-1}}$, using the specific primers listed in **Table 1**[20-22]. Total genomic deoxyribonucleic acid (DNA) was extracted using a commercial extraction kit (Bioneer Company, Korea). PCR amplification was performed in a thermocycler (Applied Biosystems, USA) as follows: initial denaturation at 96ºC for 10 min, followed by 30 cycles of 96ºC for 1 min, the annealing temperature for each primer set indicated in **Table 1** for 1 min, and 72ºC for 1 min, and a final extension step at 72ºC for 10 min. The amplification reaction was prepared in a total volume of 25µl containing 1U of Taq DNA polymerase 2X Master Mix, 1.5mM $MgCl_2$ (Ampliqon, Denmark), 0.5µM forward primer, 0.5µM reverse primer, 9µl nuclease free water, and 2.5µl DNA template (50pg concentration). PCR products were separated by agarose gel electrophoresis at 100 volts for 40 min, stained with ethidium bromide, and visualized on an ultraviolet (UV) transilluminator (UVtec, UK). The PCR products were then purified and both strands directly sequenced by Macrogen Company (Seoul, South Korea) to confirm the presence of the detected genes. Finally, online sequence alignment and analysis were conducted using the National Center for Biotechnology Information BLAST program (http://blast.ncbi.nlm.nih.gov/Blast) to compare the data found in our study with that available in the database library.

Clonal analysis by ERIC-PCR

ERIC-PCR was performed to detect any clonal relationships between the isolates. The final PCR reaction volume was 25µl and contained 2.5µl 10X PCR buffer, 0.5µl deoxynucleotide triphosphate (dNTP) Mix (10mM), 5µl $MgCl_2$, 25pM primer F, 25pM primer R, 2U Taq DNA polymerase, 3µl (50-100 ng) extracted template DNA, and 16.1µl distilled water. The amplification conditions were as follows: initial denaturation at 95ºC for 5 min, followed by 30 cycles of 94ºC for 1 min, 45ºC for 1 min, and then 72ºC for 2 min, and a final extension at 72ºC for 16 min. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Visual analysis of the ERIC-PCR profile was performed and profiles that had identical or very similar banding patterns (up to two bands different) were regarded as originating from the same DNA groups[23].

TABLE 1: Primers used for detecting metallo-β-lactamase-encoding genes in this study.

Genes	Sequence (5'→3')	Annealing temperature (°C)	Reference
bla_{IMP-1} F	ACCGCAGCAGAGTCTTTGCC	55	20
bla_{IMP-1} R	ACAACCAGTTTTGCCTTACC		
bla_{IMP-2} F	GTTTTATGTGTATGCTTCC	51	20
bla_{IMP-2} R	AGCCTGTTCCCATGTAC		
bla_{VIM-1} F	AGTGGTGAGTATCCGACAG	53	20
bla_{VIM-1} R	ATGAAAGTGCGTGGAGAC		
bla_{VIM-2} F	ATGTTCAAACTTTTGAGTAAG	52	20
bla_{VIM-2} R	CTACTCAACGACTGAGCG		
bla_{SPM-1} F	GCGTTTTGTTTGTTGCTC	53	20
bla_{SPM-1} R	TTGGGGATGTGAGACTAC		
bla_{GIM} F	TCGACACACCTTGGTCTG	52	21
bla_{GIM} R	AACTTCCAACTTTGCCAT		
bla_{SIM} F	TACAAGGGATTCGGCATCC	52	21
bla_{SIM} R	TAATGGCCTGTTCCCATG		
bla_{NDM-1} F	GGCGGAATGGCTCATCACGA	56	22
bla_{NDM-1} R	CGCAACACAGCCTGACTTTC		
oprL F	ATGAACAACGTTCTGAAATTCTCTGCT	58	17
oprL R	CTTGCGGCTGGCTTTTTCCAG		
oprL F	ATGGAAATGCTGAAATTCGGC	57	17
oprL R	CTTCTTCAGCTCGACGCGACG		

Statistical analysis

Descriptive statistics was conducted to determine frequencies. Microbiological, clinical, and demographic characteristics were assessed using the Statistical Package for the Social Sciences (SPSS)-based computer program version 16.

RESULTS

In this study, bacterial isolates were collected from 160 burn patients aged between 18 and 86 years (mean age of 52.8 ± 17.7 years), of which 115 (71.9 %) were male and 45 (28.1 %) were female. The antimicrobial resistance rates of these isolates were 100% for amoxicillin clavulanate, ciprofloxacin, and cefoxitin, 99.4% for gentamicin, amikacin, gatifloxacin, cefepime, and aztreonam, 98.8% for imipenem, 98.1% for meropenem, 84.4% for piperacillin/tazobactam, 80% for ceftazidime, and 0% for colistin and polymyxin B (0%). All 160 isolates were considered multidrug-resistant (MDR) as they were intermediately or fully resistant to at least three different classes of antimicrobial agents, including β-lactams, aminoglycosides, and fluoroquinolones.

In total, 158 (98.8%) isolates were non-susceptible to these carbapenems, of which 69 (43.7%) were found to be MBL producers **(Figure 1)**. Twenty-eight (17.5%) isolates encoded bla_{VIM-1}, which was the most commonly encoded gene among those investigated, followed by the bla_{IMP-1} (15.6%) and bla_{SPM-1} (5.6%) genes either alone or in combination. The bla_{IMP-1} gene coexisted with bla_{VIM-1} in 2 (1.3%) isolates. The bla_{IMP-2}, bla_{VIM-2}, bla_{GIM}, bla_{SIM}, and bla_{NDM-1} genes were not detected among the isolates assessed in this study.

ERIC-PCR revealed that the MBL-producing *P. aeruginosa* belonged to three distinct genotypes, A (53/69, 76.8%), B (14/69, 20.3%), and C (2/69, 2.9%), indicating clonal dissemination of these resistant isolates occurred in this referral burn hospital. As shown in **Table 2**, the bla_{VIM-1} gene was the MBL gene most frequently detected among the isolates and was found among the MBL-producing isolates with the A (17/69, 24.6%), B (7/69, 10.1%), and C genotypes (2/69, 2.9%). Meanwhile, bla_{IMP-1}-positive isolates were only found with genotypes A (18/69, 26.1%) and B (5/69, 7.2%).

TABLE 2: ERIC-PCR results for metallo-β-lactamase-positive *Pseudomonas aeruginosa* isolates.

Genes	Isolates			
	Type A	Type B	Type C	Total
	N (%)	N (%)	N (%)	N (%)
bla_{VIM-1}	17 (24.6)	7 (10.1)	2 (2.9)	26 (37.7)
bla_{IMP-1}	18 (26.1)	5 (7.2)	-	23 (33.3)
bla_{SPM-1}	7 (10.1)	2 (2.9)	-	9 (13)
bla_{VIM-1} + bla_{IMP-1}	2 (2.9)	-	-	2 (2.9)
NO MBLS genes	9 (13)	-	-	9 (13)
Total	**53 (76.8)**	**14 (20.3)**	**2 (2.9)**	**69 (100.0)**

ERIC-PCR: Enterobacterial repetitive intergenic consensus-polymerase chain reaction.

DISCUSSION

Patients with burn injuries face a higher risk of contracting nosocomial infection due to having partial or complete loss of the skin barrier and a weakened immune system that makes delivery of immediate specialized care a necessity to reduce the rates of mortality and morbidity[24]. *P. aeruginosa* is a leading cause of nosocomial infections that frequently occur in patients undergoing long-term hospitalization and, particularly, those admitted to burn units. Antimicrobial treatment of infections caused by this organism in a patient with burns is often complicated by the appearance of resistance to highly effective antibiotics, such as carbapenems[1]. During the last decade, acquired MBL-carrying *P. aeruginosa* isolates with the potential to efficiently hydrolyze carbapenems and most β-lactam drugs have emerged[8].

The present study uncovered a significantly high level of resistance to routinely prescribed antibacterial agents in our target hospital. All isolates were MDR and, among the antimicrobials tested, were fully resistant to amoxicillin clavulanate, ciprofloxacin, and cefoxitin. Moreover, more than 90% of the isolates were resistant to gentamicin, amikacin, gatifloxacin, cefepime, aztreonam, imipenem, and meropenem, whereas colistin and polymyxin B were the most active antimicrobial agents against our *P. aeruginosa* isolates. Overall, the frequency of MDR isolates in the present study was higher than in recent studies carried out by Asadpour et al. on *P. aeruginosa* isolates from patients with burns in Northern Iran (72.2%)[25], and Rostami et al. in southwest of Iran in 2018 (72%)[26]. In total, these results indicate that the available choices for appropriate treatment of infection caused by these resistant isolates are currently limited. However, the data on colistin and polymyxin B used in our study were comparable to those in other studies[27-29] and, therefore, these are considered the antibiotics of choice against *P. aeruginosa* strains isolated from the patients with burn infections in this hospital. The presence of higher rates of resistance against the antimicrobial agents tested in the current study reflects the irrational and extensive administration of these antibiotics in our medical settings, which eventually resulted in the emergence of this resistance among bacterial isolates. Furthermore, our results highlight the necessity for launching local and nationwide surveillance guidelines on monitoring antibiotic administration to prevent antimicrobial resistance and

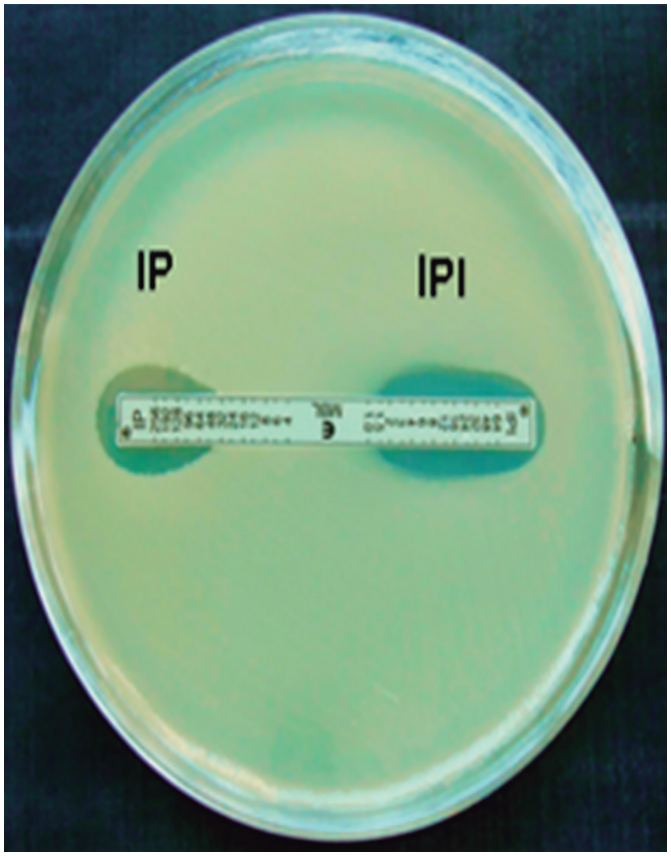

FIGURE 1: Metallo-β-lactamase E-test results for a metallo-β-lactamase-positive *Pseudomonas aeruginosa* isolate. This figure presents a decreased imipenem (IP) maximum inhibitory concentration from 32 to 1.5μg/ml upon constant exposure to EDTA (IPI). This value is less than the cut-off value of 8.0, resulting in this isolate being classified as a metallo-β-lactamase positive *P. aeruginosa* isolate. **IP:** Imipenem; **IPI:** imipenem-inhibitor; **EDTA**: ethylenediaminetetraacetic acid.

the occurrence of resistance against antibiotics among bacterial agents in this major referral burn center in Tehran.

In the current study, 158 (98.8%) isolates were non-susceptible to the carbapenems used. Among those, 69 (43.7%) isolates were MBL producers based on the MBL E-test. This frequency was higher than in other studies, such as in one report from India[29] and two other recent studies from Iran[30,31].

This study found 28 (17.5%) isolates carried $bla_{\text{IMP-1,}}$ which was the most common MBL gene, followed by the genes $bla_{\text{VIM-1}}$ (15.6%) and $bla_{\text{SPM-1}}$ (5.6%), either alone or in combination. In two similar studies from Iran, Lari et al.[31] and Salimi et al.[32] reported the presence of $bla_{\text{VIM-1}}$ and $bla_{\text{IMP-1}}$ genes among *P. aeruginosa* isolates obtained from patients with burns. In another study, Saffari et al. found that 27 (18%) and 8 (5.5%) of isolates were positive for the $bla_{\text{VIM-1}}$ and $bla_{\text{VIM-2}}$ genes, respectively[33]. Radan et al. showed 74.3% of the MBL isolates in their study carried the bla_{IMP} gene[27]. In eastern Algeria, Meradji et al. stated that 46.7% of *P. aeruginosa* isolates from burn patients were MBL producers and contained the $bla_{\text{VIM-4}}$ and $bla_{\text{VIM-2}}$ genes[34]. We previously assessed the presence of the $bla_{\text{IMP-1}}$ and $bla_{\text{VIM-1}}$ genes within *P. aeruginosa* and *Acinetobacter baumannii* clinical isolates collected from medical centers in Iran[35,36]. Overall, our new findings highlight the widespread presence of the $bla_{\text{IMP-1}}$ and $bla_{\text{VIM-1}}$ genes in nosocomial *P. aeruginosa*, which contributes to numerous unpleasant health care-associated infections. To our knowledge, this is the first report on the existence of $bla_{\text{SPM-1}}$-related MBL genes among *P. aeruginosa* clinical isolates collected from patients with burns in Iran. In our present study, the $bla_{\text{IMP-1}}$ and $bla_{\text{VIM-1}}$ genes coexisted in 2 (1.3%) isolates. The co-existence of similar resistance genes in *P. aeruginosa* clinical isolates has been reported in a previous study in Iran[32]. The genes encoding these enzymes are often located on transferable genetic platforms and can potentially spread to other bacterial species through horizontal gene transfer. Therefore, appropriate infection control guidelines and treatment protocols are needed to stop the further dissemination of these resistance genes to other bacterial agents in medical centers. In our study, phenotypic tests yielded different positive results when screening for MBL-producing *P. aeruginosa* isolates compared to molecular methods. In total, 87% of isolates were confirmed to carry MBL genes by the PCR method. This discrepancy between the results obtained by PCR and MBL E-test could be due to the membrane permeabilizing effect of EDTA, which could increase the susceptibility of *P. aeruginosa* and *Acinetobacter* spp. to antibacterial agents[37].

The ERIC-PCR results revealed that MBL-producing *P. aeruginosa* belonged to three separate clones, indicating clonal dissemination of these resistant isolates occurred in this referral burn center. The A genotype was the most common (76.8%) found in this study and was strongly associated with the clonal spread of resistant isolates and patient-to-patient transmission.

In conclusion, the results of this study revealed a noticeably high prevalence of MBL-producing *P. aeruginosa* isolates in our hospital. MBL-encoding genes are often carried by mobile genetic elements that can rapidly spread between different strains through horizontal gene transfer. However, early recognition of MBL-producing isolates, establishing comprehensive guidelines and infection control measures, and employing an all-inclusive protocol for antimicrobial therapy based on laboratory data are necessary to significantly decrease further dissemination of these resistant pathogens in our medical settings.

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Conflict of interest

The authors declare that there is no conflict of interest.

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