Original Article

Prevalence of IMP and VIM Metallo-Beta-Lactamases in *Pseudomonas aeruginosa* Isolates from Clinical and Environmental Specimens in Intensive Care Units (ICUs) of Rasht Hospitals, Iran

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Introduction: *Pseudomonas aeruginosa* is one of the leading causes of nosocomial infections, and antibiotic resistance of this pathogen is an important concern in treating such infections. The current work was conducted to investigate the prevalence of *bla-IMP,* and *bla-VIM* metallo-beta-lactamase (MBL) among clinical and environmental *P. aeruginosa* isolates obtained from ICUs of different hospitals in Rasht, Iran. **Methods:** A total number of 35 *P. aeruginosa* strains including 20 clinical and 15 environmental strains were isolated from ICUs. The isolated bacteria were screened for MBL production using Combined Disc Synergy Testing (CDST) assay. The frequency of *bla-IMP* and *bla-VIM* among MBL producing *P. aeruginosa* (MBL-PA) was investigated using Polymerase Chain Reaction (PCR). Also, the antibiotic susceptibility of all isolates was determined. **Results:** According to the results, 51% of isolates were regarded as MBL-PA while *bla-IMP* or *bla-VIM* genes were detected in 37% of isolates. The environmental isolates showed higher resistance to the majority of antibiotics compared to the clinical isolates, and MBL genes were more prevalent among environmental isolates. **Conclusion:** Higher resistance of environmental *P. aeruginosa* strains in ICUs shows a need to pursue newer approaches, including novel cleaning methods and surveillance programs, to reduce nosocomial infections. *J Med Microbiol Infec Dis, 2015, 3 (3-4): 62-66*.

Keywords: Carbapenems, Antibiotic resistance, Metallo-Beta-Lactamase, Nosocomial infection.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen which is a leading cause of infections particularly among immunocompromised patients, burn patients as well as cystic fibrosis (CF) patients [1]. Owing to the intrinsic and acquired antibiotic resistance, infections caused by *P. aeruginosa* is notably challenging. Among Beta-lactams, carbapenems have shown high potency against *P. aeruginosa* [2]. Metallo-Beta-Lactamases (MBLs) are emerging group of enzymes which efficiently hydrolyse all beta-lactams including carbapenems. MBLs are mainly encoded by the *bla-IMP* and *bla-VIM* genes which readily spread among bacteria [3].

MBL producing *P. aeruginosa* (MBL-PA) associated with infections have emerged recently particularly in Intensive Care Units (ICUs) [4-5]. Although several studies on MBL-PA nosocomial infections have been performed in Iran [6-7], few epidemiological studies on MBL-PA associated infection in ICUs have been reported. Also, environmental reservoirs of *P. aeruginosa* in ICUs has rarely been investigated in Iran.

The current descriptive study was conducted to investigate the prevalence of *bla-IMP* and *bla-VIM* genes among MBL-PA isolated from clinical and environmental samples from ICUs from Rasht city, Iran and to determine antibiotic resistance profile of the isolates.

MATERIAL AND METHODS

Bacterial isolates. *P. aeruginosa* strains were isolated from ICUs of three different hospitals in Rasht city, Iran from February 2015 to May 2016. Clinical isolates were obtained from patients within an age range of 18 and 61 years (mean age= 37.9 years). Eight strains were isolated from females (with a mean age of 38.1 years), and the remaining were obtained from males (with a mean age of 44.9 years). Patient specimens including respiratory secretions, urine, wound swabs and blood, were collected based on clinical need. Environmental samples were collected from bed equipment (bed rails, bed sheets, blankets), hospital equipment (ventilators, suction devices, sinks, and showers) and stuff equipment (gloves, masks, glasses, stethoscopes, and sphygmomanometers). The swabs were kept in transport media and sent to the microbiology laboratory. Blood agar and Eosin Methylene Blue (EMB) media (Merck) were used to isolate bacterial strains. Bacterial identification was performed based on the biochemical and morphological assays using Hi-Media bacterial identification kit (India). The isolated *P. aeruginosa* were kept at -70°C in Tryptic Soy Broth (TSB) containing 15% glycerol for further analysis.

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Antimicrobial susceptibility testing. Antimicrobial susceptibility of the bacterial isolates was investigated using Kirby-Bauer disc diffusion assay according to the Clinical Laboratory Standard Institute (CLSI) recommendation [8]. The antimicrobial discs were as following: piperacillin/tazobactam (100/10µg), amikacin (30µg), ceftazidime (30µg), Ciprofloxacin (5µg), Gentamicin (10µg), meropenem (10µg) and imipenem (10µg). *P. aeruginosa* ATCC 27853 was used as the control strain for susceptibility testing.

Screening of MBL-PA. Bacterial strains which were resistant to carbapenems (imipenem and meropenem) as well as the isolates which showed resistance to cephalosporins (owing to ESBLs) were submitted for subsequent testing using Combined Disc Synergy Test (CDST). Briefly, bacterial suspension of each bacterial isolate (1.5×10^8 CFU/mL) was prepared and inoculated on the surface of a Muller Hinton Agar (MHA) plate. Then, imipenem disc (10µg) and the combined disc containing imipenem/EDTA (10µg/100mM) were placed on the surface of MHA, and the plates were incubated overnight at 37°C. Then, inhibition zone diameter around the combined disc for every isolate was determined and compared to the inhibition zone diameter of imipenem disc. Any strain with an increase in the inhibition zone diameter greater than 7 mm around combined disc was regarded as MBL-PA [8].

Molecular detection of *bla-IMP* and *bla-VIM*. Molecular detection of *bla-IMP* and *bla-VIM* genes among MBL-PA isolates was performed using Polymerase Chain Reaction (PCR). Bacterial DNA extraction was performed using CinnaGen® DNA extraction kit according to the manufacturer's protocol. Briefly, the isolates were grown in Luria broth (Merck, Germany) overnight at 37ºC, centrifuged at 8000×g for 10 min. The resulting pellet was re-suspended in 200µL prelysis buffer and 20µL ributinase. Then, 400µL of lysis buffer was added and incubated for 1-2 min at room temperature to lyse bacterial cells. The cell lysates were centrifuged at 10000 g for 5 min and DNA was precipitated by 300µL precipitation buffer and purified using spin columns.

The master mix for the PCR was prepared as follows: 2.5µL of 10× PCR buffer, 1µL of 25mM $MgCl_2$, 1µL of 10mM dNTP mix, 0.5µL of Taq DNA Polymerase, 18µL of MilliQ water and 0.5µL of each of the forward and reverse primers. Finally, 1µL of each DNA template was added to the corresponding tubes to make up the final reaction volume of 25µL. The PCR primer pairs used in this study were designed using Gen runner, and Oligo7 softwares (Table 1).

The amplification reactions consisted of an initial DNA denaturation at 95ºC for 4 min followed by 30 cycles of denaturation at 94ºC for 30 s, primer annealing at 60ºC for 1 min for both *bla-IMP* and *bla-VIM* and polymerization at 72ºC for 1 min. The polymerization was concluded by an extension period of 8 min at 72ºC. Then, PCR products were mixed with 3µL PowerLoad™ DNA stain and were visible after electrophoresis in a 1% agarose gel and under UV trans-illumination.

The amplified genes were sequenced (Macrogen, South Korea) and generated sequences were submitted to the GenBank (NCBI) and blasted with other published sequences from the GenBank database.

Statistical analysis. Statistical analyses were carried out using SPSS 18.0 software and χ^2 test. A *P* value of 0.05 was considered as statistically significant.

RESULTS

A total number of 35 *P. aeruginosa* including 20 clinical and 15 environmental isolates were included in this study. The clinical isolates were obtained from respiratory secretions (50%), urine (40%), blood (10%) and wounds (5%), while environmental *P. aeruginosa* were isolated from the bed, stuff and hospital equipment with the frequency of 21%, 33%, and 46%, respectively.

Antibiotic susceptibility profile. Higher susceptibility to almost all antimicrobials was observed among clinical isolates compared to the isolates from environmental samples. The most effective antibiotics against clinical isolates were amikacin, gentamicin, and ciprofloxacin with susceptibility of 100%, 96%, and 88%, respectively. Among environmental isolates, 87% were susceptible to amikacin while the susceptibility to gentamicin, ciprofloxacin, piperacillin/tazobactam and imipenem was 33%. Also, although no resistance to ceftazidime was observed among clinical isolates, 67% of environmental isolates were resistant to this antibiotic. Also, MBL-PA were significantly more resistant than non-MBL-PA to various antibiotics. Antibiotic susceptibility of the isolates is presented in figures 1 and 2.

Screening of MBL-PA. Screening of MBL-PA was performed on the isolates which displayed resistance to carbapenems and/or cephalosporins using CDST assay. The result revealed that 18 isolates including 8 clinical isolates (40%) and 10 environmental isolates (67%) showed increased zone of inhibition (ZOI) around IMP/EDTA discs compared to the IMP disc alone (Fig. 3). These isolates were regarded as MBL-PA strains which were further analyzed using molecular assay.

Table 1. Primer pairs used for amplification of MBL genes

Genes	Primer sequence	Product size
BLA-IMP	F:5'GGCATAGAGTGGCTTAATTCTC3'	250 bp
	R: 5'GGCCAAGCTTCTATATTTGC3'	
BLA-VIM	F:5'CAAATTGGACTTCCTGTAACG3'	373 bp
	R:5'TATAGAGGTGGGCCATTCAG3'	

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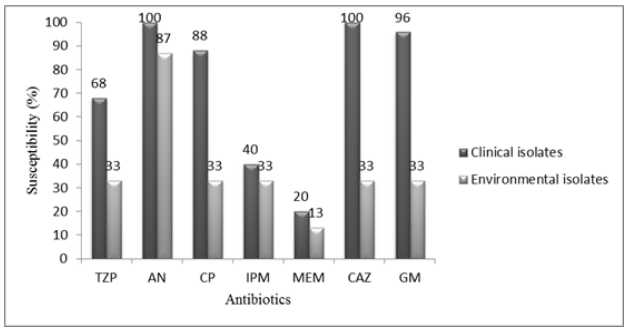


Fig. 1. Antibiotic susceptibility of clinical and environmental *P. aeruginosa* isolates. TZP: Piperacillin/Tazobactam, AN: Amikacin, CP: Ciprofloxacin, IPM: Imipenem, MEM: Meropenem, CAZ: Ceftazidime, GM: Gentamicin

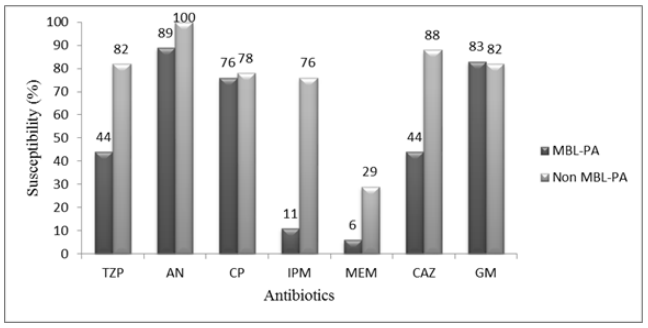


Fig. 2. Antibiotic susceptibility of MBL-PA and non MBL-PA isolates. TZP: Piperacillin/Tazobactam, AN: Amikacin, CP: Ciprofloxacin, IPM: Imipenem, MEM: Meropenem, CAZ: Ceftazidime, GM: Gentamicin

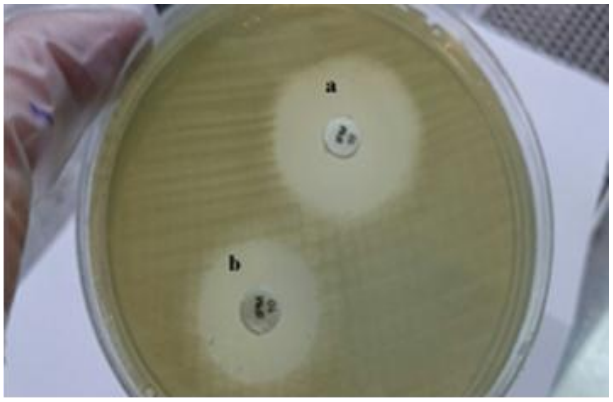


Fig. 3. Phenotypic screening of MBL-PA. a, IMP/EDTA disc; b, IMP disc. Strains with increased inhibition zone more than 7 mm are regarded as MBL-PA

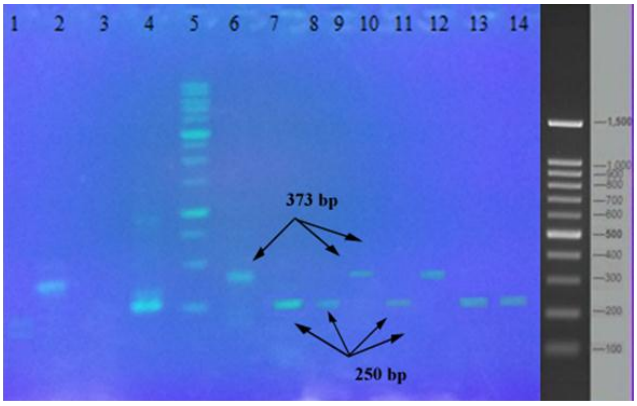


Fig. 4. PCR amplification of *bla-IMP* and *bla-VIM* genes. Lanes1, 3, negative controls; lane 2, positive control for *bla-VIM*; lane 4, positive control for *bla-IMP*; lane 5, 100 bp DNA ladder; lanes 6-14, bacterial isolates

Molecular investigation of *bla-IMP* and *bla-VIM*. According to the molecular assay, a total number of 13 *P. aeruginosa* isolates harbored either *bla-IMP* or *bla-VIM* genes. Among clinical isolates, *bla-IMP* was detected in five isolates while and *bla-VIM* was detected in one strain. Also, *bla-IMP* and *bla-VIM* genes were observed in five and two environmental isolates, respectively. Neither of the isolates harbored both genes (Fig. 4).

DISCUSSION

In this study, the prevalence of MBL-PA strains among clinical and environmental specimens from different ICUs was investigated. Our results showed that hospital equipment and stuff equipment, (where, 46% and 32% of the environmental strains were isolated, respectively) were the most important reservoirs of *P. aeruginosa* in ICUs. Thus, the ICU stay, itself is not the primary cause of the infection and infection usually occurs by hospital equipment and health stuff. Respiratory and urinary tract infections were more prevalent among ICU patients, which could be associated with secure transmission of the infectious agent via hospital and stuff equipment to the patients. High prevalence of respiratory and urinary tract infection by *P. aeruginosa* has been reported previously by several authors [9-11].

Clinical isolates were susceptible to most antibiotics including amikacin and gentamicin. However, they displayed low susceptibility to imipenem and meropenem. This finding shows that MBL-PA should be considered in case the patients are not responsive to carbapenem therapy. MBL-PA were significantly more resistant than non-MBL-PA to various antimicrobial agents, except for piperacillin/tazobactam. The multi-drug resistant phenotype is expected in MBL producers, as they hydrolyse all beta-lactams except aztreonam, and are associated with gene cassettes harboring different resistance genes [12].

Surprisingly, higher resistance to almost all studied antibiotics was observed among environmental isolates compared to the clinical strains. The environmental isolates are mostly associated to biofilms which facilitate genetic material exchange between bacteria and prevents penetration of antimicrobials, resulting in higher antibiotic resistance. Also, in the hospital environment, bacterial strains are constantly exposed to a variety of antimicrobials which may enhance bacterial resistance mechanisms. Thus, we postulated that the higher resistance of environmental *P. aeruginosa* could be associated with the higher potential of biofilm formation and enhanced antimicrobial mechanisms. The high resistance of environmental *P. aeruginosa* has been described previously [13-14].

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Resistance to carbapenems was observed among both clinical and environmental strains (71%), while 51% of isolates were screened as MBL-PA using CDST assay. This finding shows that, in addition to the MBL production, several resistance mechanisms are involved with carbapenem resistance among *P. aeruginosa* strains. Increased expression of efflux systems, reduced porin expression, and increased activity of chromosomal cephalosporinase have been defined as contributory factors to carbapenems resistance [15].

According to our results, 13 isolates harbored *bla-IMP* or *bla-VIM* genes while 18 strains were positive by CDST assay. The difference could be due to the lower sensitivity of phenotypic screening test compared to the molecular assay and involvement of other MBL including NDM (New Delhi metallo-ß-lactamase) and SPM (São Paulo metallo-ß-lactamase) types MBL, which needs more investigation [16].

Higher prevalence of *bla-IMP* was observed among both clinical (25%) and environmental (33%) isolates, while *bla-VIM* was observed with the frequency of 5% and 13% among clinical and environmental strains, respectively. Previous studies reported moderate to high level of MBL frequency among *P. aeruginosa* associated with nosocomial infections. Perez *et al.*, in a study on Cystic Fibrosis (CF) patients, reported that 21% of the *P. aeruginosa* were MBL producers and *bla-IMP* was the most prevalent type which was similar to our finding [16]. Also, they found an association between biofilm formation and MBL production among *P. aeruginosa* nosocomial isolates which could describe the higher resistance of MBL-PA compared to non-MBL-PA strains.

Khosravi *et al.*, in a study on burn patients in Ahvaz (Iran) found that 66% of the *P. aeruginosa* isolates were imipenem susceptible which was higher than our finding [7]. Also, unlike to our study they reported that all MBL-PA harbored *bla-VIM*. In another study, similar to our finding, Bahar *et al.* in a study on burn patients in Tehran, found that 38% of *P. aeruginosa* isolates were imipenem susceptible and they also reported that all MBL-PA harbored *bla-VIM* [6]. Conversely, we found that *bla-IMP* was more prevalent than *bla-VIM*. The difference could be due to the different geographical regions as well as different patient type.

In conclusion, hospital environments are potential reservoirs for nosocomial infections, which calls for an intervention program to reduce environmental transmission of pathogens. Although high resistance to carbapenems was observed among *P. aeruginosa* isolates, the moderate prevalence of MBL-genes was observed indicating the involvement of variety resistance mechanisms in resistant to carbapenems. Also, our study showed that environmental isolates were significantly more resistant to the majority of antibiotics which shows the proper disinfection strategies are essential to reduce nosocomial infection.

In our study, we tried to explain possible differences between clinical and environmental *P. aeruginosa* isolated from ICUs. However, further studies including molecular phylogenetic analysis are required to elucidate the genetic lineage of the clinical and environmental strains isolated from different hospitals.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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