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Detection of *Pseudomonas aeruginosa* Producing Metallo β-Lactamases (VIM, SME, AIM) in the Clinical Isolates of Intensive Care Units of Al-Zahra Hospital in Isfahan, Iran

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ABSTRACT

***Background*:** *Pseudomonas aeruginosa* is a severe challenge for antimicrobial therapy, because of chromosomal mutations or exhibition of intrinsic resistance to various antimicrobial agents such as most β-lactams. We undertook this study to evaluate the existence of SME, AIM and VIM metallo-beta lactamases encoding genes among *P. aeruginosa* strains isolated from ICU patients in AL-Zahra Hospital in Isfahan, Iran.

***Methods*:** In a retrospective cross sectional study that was conducted between March 2012 to April 2013, in total 48 strains of *P. aeruginosa* were collected from clinical specimens of bedridden patients in ICU wards. Susceptibility test was performed by disc diffusion method. All of the meropenem resistant strains were subjected to modified Hodge test (MHT) for detection of carbapenemases. Multiplex PCR was performed for detection of *VIM*, *blaAIM*, *blaSME* genes.

***Results*:** In disk diffusion method imipenem and meropenem showed the most and colistin the least resistant antimicrobial agents against *P. aeruginosa* strains. Of the 48 isolates 36, (75%) were multidrug resistant. Amplification of β-lactamase genes showed the presence of *blaVIM* genes in 7 (%14.6) strains. All of the isolates were negative for *blaSME* and *blaAIM* genes. We couldn't find any statistically significance difference among presence of this gene and MDR positive, age or source of the specimen.

***Conclusion*:** As patients with infections caused by MBL-producing bacteria are at an intensified risk of treatment failure, fast determination of these organisms is necessary. Our findings may provide useful insights in replace of the appropriate antibiotics and may also prevent MBLs mediated resistance problem.

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Introduction

Pseudomonas aeruginosa is a common Gram-negative bacillus associated with difficult nosocomial infections (1). This bacterium is particularly found in intensive care units (ICUs) and also in immunocompromised and critically ill patients (2). *P. aeruginosa* is a severe challenge for antimicrobial therapy, because of chromosomal mutations or exhibition of intrinsic resistance to various antimicrobial agents such as most β-lactams (1). Acquired resistance determinants in *Pseudomonas* spp., may be related either to low bacterial outer membrane permeability (such as loss or modification of the OprD2 porin or over expression of efflux pumps), over expression of β –lactamases dominating no significant carbapenemase activity (AmpCs), or to expression of really carbapenemases (3). Carbapenemase producing is one of the most important mechanisms because it was associated with higher mortality rates compared with non-carbapenemase-producing (4). Carbapenemases belong to various Ambler classes, containing A, the serine-based penicillinases (e.g. KPC, GES and SME types), B, the metallo-β-lactamases (MBLs such as IMP, VIM, AIM, SIM, DIM, and NDM types), and D, the oxacillinases (e.g. OXA-23, -24, -48 and -58) (5). In *Pseudomonas* spp, the most carbapenemases are mainly MBLs (3).The reported carbapenemases in *P. aeruginosa* included Verona integron-encoded β- lactamase (VIM), Adelaide imipenemas (AIM) , Serratia marcescens enzyme (SME) , non metallo-enzyme carbapenemase (NMC), Serratia marcescens enzyme (SME), Klebsiella pneumonia carbapenemase (KPC), imipenem hydrolyzing b-lactamase (IMI), Guiana extended-spectrum b-lactamase (GES), imipenemase (IMP), German imipenemase (GIM), Sao Paulo metallo-β-lactamase (SPM), New Delhi metallo-β-lactamase (NDM), and oxacillinase-48 (OXA-48) (4). Resistance to carbapenems are included the production of Ambler class D and B β-lactamases, also referred to as metallo-beta-lactamases (MBL) (6). Carbapenemase is a critical problem because of their resistant to nearly all β - lactams. Besides, the initial identification of carbapenemases is essential because carbapenemase genes are generally found on plasmids, leading to their fast clonal spread (7). P. aeruginosa producing MBLs was first described from Japan in 1991 (1) and since then the prevalence of these nosocomial strains has been increased all over the world such as Asia, Europe, Australia, South America and North America (8). Current different surveillance researches from the USA and Europe have been showed an increase from 4% in the 1990s to 14-30% in the 2000s in prevalence of carbapenem-resistant *P. aeruginosa* isolates, also MBL-producing *P. aeruginosa* isolates constitute 20-42% of all nosocomial isolates (4).Several previous studies from different parts of Iran reported a high rate of occurrence of MBL producing *P. aeruginosa* from various hospital units (2). With regard to the high mortality rates of these multidrug resistance infections (ranging from 18% to 61%), carbapenems including imipenem and meropenem, are considered crucial for treating of many *P. aeruginosa* infections (4). Screening of carbapenemase producers among carbapenem resistant *P. aeruginosa* isolates is significant. Some phenotypic techniques for in vitro detection of carbapenemase production, such as the modified Hodge test (MHT), has been widely performed by routine labs because it directly analyzes the carbapenemase activity of a tested strain. Because of its simpleness, the CLSI has recommended it for Enterobacteriaceae with raised carbapenem MICs or reduced disk diffusion inhibition zones (9). MHT is not adequately sensitive, specific and do not provide a detailed molecular recognition of a carbapenemase that is required for epidemiological studies and infection control purposes. Therefore molecular identification of carbapenemase genes is an important alternative for an exact, rapid and sensitive determination of carbapenemase genes (10). The multiplex PCR technique is one of the most current and reliable techniques for the detection and confirmation of carbapenemase

producing *P. aeruginosa*. This method could allow one to recognize more than two related carbapenemase genes in a single test (10, 11).

Even so, the detection of carbapenemase producers must practically be followed by a rapid adjustment of the antibiotic therapy and by the isolation of colonized patients in order to stop the development of nosocomial outbreaks (3). In current years because of the potential relevance of development of microbial resistance, rapid identification of MBL producing Gram-negative bacilli especially *P. aeruginosa* is essential for the optimum treatment of patients particularly in critically ill hospitalized patients (12). There is not much data available on MBL producing *P. aeruginosa* isolates from Iran. Therefore, we undertook this study to evaluate the existence of SME, AIM and VIM metallo-beta lactamases encoding genes among *P. aeruginosa* strains isolated from ICU patients in AL- Zahra Hospital in Isfahan, Iran.

Material and method

Sampling and bacterial isolation

In a cross-sectional study, 48 *P. aeruginosa* isolates were collected from clinical specimens of bedridden patients in ICU wards of Al-Zahra hospital of Isfahan city (March 2012 to April 2013). These isolates were obtained from culture of specimens from tracheal aspirate, blood, urine, wound, catheter and peritoneal fluid of the patients. Bacteria were determined as *P. aeruginosa* by biochemical tests or if they had following characteristics: gram-negative bacilli, citrate positive, non- fermentative, TSI Alk/Alk, motile, H2S negative, urease negative, oxidase positive, and catalase positive. The confirmed isolates were kept preserved at -70 °C. Standard strain *P. aeruginosa* ATCC 27853 was used as control (13).

Antimicrobial susceptibility tests

Antibiotic susceptibility testing of *P. aeruginosa* isolates was performed by the disc diffusion method on Mueller-Hinton agar (Merck, Germany). Susceptibility was defined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (14). Antibiogram discs containing meropenem (MEM 10 µg), imipenem (IPM 10 µg), ceftazidime (CAZ 30 µg), cefepime (FEP 30 µg), amikacin (AK 30 µg), ciprofloxacin (CIP 5 µg), colistin sulphate (10 µg), ampicillin/ sulbactam (20 µg) and cefotaxime (CTX 30µg) discs (MAST, Bootle, and Merseyside, UK). *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used as reference strains in susceptibility test (4, 13).

Modified hodge test

All of the meropenem resistant strains (zones i.e 16mm-21mm) were subjected to modified Hodge test (MHT) for detection of carbapenemases. Besides combined disk tests with boronic acid for the recognition of class a carbapenemases or with EDTA for the detection of metallo β lactamases. The existence of a zone of inhibition due to carbapenemase production by the test strain was considered as positive (15, 16).

Extraction of total DNA

For molecular detection, the total DNA content of each isolate was extracted with boiling method. In brief a single colony from a 16 h culture on the nutrient agar (Hi-Media, India) was suspended in 50 µl of TES (50 mM Tris hydrochloride (pH 8.0), 5 mM EDTA, 50 mM NaCl), and the suspension was heated at 95 °C for 10 min and centrifuged at 15,000 × g for 2 min. DNA containing supernatant was transferred to new sterile DNase free-RNase free micro tubes (17).

Rapid detection of VIM, SME and AIM genes by multiplex PCR

Multiplex PCR was performed for amplification of *P. aeruginosa* VIM, SME and AIM genes, using the primers listed in Table 1 according to the previous protocols (18). Two microliters of total

DNA was included to multiplex PCR in a 50µL reaction mixture.

The mix for the detection of *blaIMP*, *blaAIM*, and *blaSME* genes contains 1× PCR buffer (10 mmol/L Tris–HCl (pH 8.3), 1.5 mmol/L of MgCl2, 0.125 mmol/L of each deoxynucleotide triphosphate, 10 µmol/L of each primer, and 2 U of AmpliTaq Polymerase (Fermentas R, Korea). Amplification was carried out with the following thermal cycling conditions: Initial DNA denaturation at 94 °C for 10 minutes, then 36 cycles of denaturation at 94°C for 30 seconds, annealing at 52 °C for 40 seconds and extension at 72 °C for 50 seconds, followed by final extension at 72 °C for five minutes . Agarose gel electrophoresis of the amplified DNA with 100 bp size marker (Fermentas R, Korea) were done for one hours at 80 V in a 2% agarose gel in 1× TAE (40 mmol/L Tris–HCl (pH 8.3), 2 mmol/L acetate, 1 mmol/L EDTA) containing 0.05 mg/L ethidium bromide to detect the specific band (18).

Statistical analysis

For the statistical analyses, the statistical software SPSS version 18.0 for Windows (SPSS Inc., Chicago, IL) was utilized. Continuous variables were described as mean ± standard deviation (SD) and compared using the Student t test, or described as the median as well as range, Categorical variables were compared with a Chi-square test or Fisher exact test if the expected values were less than 10.All tests were two-tailed and $p < 0.05$ was considered statistically significant.

Result

Demographic characterization of carriages of Pseudomonas aeruginosa

The medical records of the 48 index patients were reviewed. The mean age was 49.5±19.2 years, with a male to female ratio of 1.82:1. 17 (35.4%) of them were female and 31 (64.6%) were male. The specimens included urine (n=19, 39.5%), blood (n= 12, 25%), tracheal aspirate (n=11, 22.9%), wound (n=4, 8.3%), catheter (n=1, 2.08%) and peritoneal fluid (n=1, 9%). The most common underlying diseases were urinary and blood infections.

Antimicrobial susceptibilities of clinical strains

The antibiotic resistance of these strains is shown in figure 1. In disk diffusion method imipenem and meropenem showed the most and colistin the least resistant antimicrobial agents against *P. aeruginosa* strains. Susceptibility rates of the isolated *P. aeruginosa* strains in different samples are represented in Figure 1.

Of the 48 isolates 36, (75%) were multidrug resistant. Our result didn't show any significant relation between the MDR positive and MDR negative isolates and gender using chi square analysis (p=0.99). Also, difference between MDR and non MDR isolates with source of the specimen and mean age were not statistically significance (p>0.05). Frequency of MDR and non MDR isolates with demographic characteristics of patients is listed in Table 2.

Bacterial isolates tested for the presence of VIM, AIM and SME genes

PCR screening for the presence of *blaVIM* gene revealed that 7 (14.6%) isolates carry carbapenemase-producing genes, which all belonged to the VIM family (Figure. 2). We couldn't find any statistically significance difference among Presence of this gene and MDR positive (p=0.11), mean age (p=0.64) or source of the specimen (p=0.15). Prevalence of *blaVIM* gene in male and female patient was 16.1% and 11.8% respectively. Comparing the sex group and presence of this gene by chi square analysis didn't show any statistically significance difference (p>0.05). Also PCR amplification showed that all of the isolates were negative for *blaSME* and *blaAIM* genes.

Table 1. Platelet counting in 0.5 ml platelet+0.02 gram polysaccharide.

No **(bp)** [a]	Gene	Primer sequence 5´-3´	Product size	Ref.
Primer 1	*blaAIM*	F- CTGAAGGTGTACGGAAACAC R- GTTCGGCCACCTCGAATTG	322	3
Primer2	*blaVIM*	F-GATGGTGTTTGGTCGCATA R- CGAATGCGCAGCACCAG	390	3
Primer3	*blaSME*	F- ACTTTGATGGGAGGATTGGC R- ACGAATTCGAGATCACCAG	551	3

a Nucleotide numbering begins at the initiation codon of genes.

Figure 1. Antimicrobial resistance rates among *Pseudomonas aeruginosa* isolates.

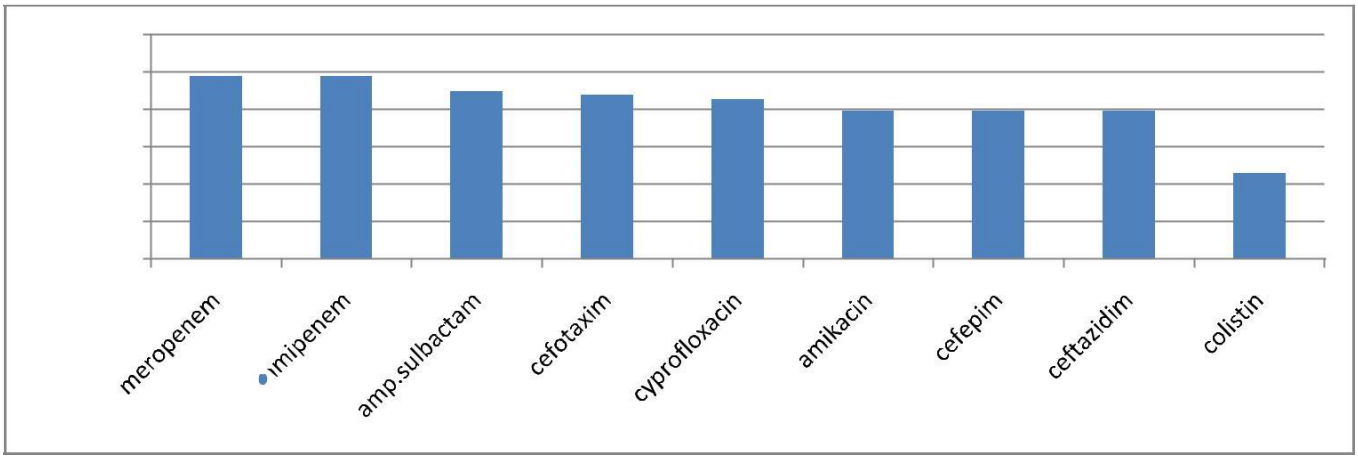

Table 2. Frequency of MDR and non MDR isolates according to demographic characteristics of patients.

Parameters	MDR No. (%)	Non MDR No. (%)	P
Age, mean ± SD (y) 49.1±17.4	50.4±24.8		0.85
Sex			
Male	23(74.2)	8(25.8)	0.99
Female	13(76.5)	4(23.5)	
Isolated specimen site			
Catheter	1 (100)	0 (0)	
Urine	14 (73.7)	5 (26.3)	
Tracheal aspirate	6 (54.5)	5 (45.5)	
Blood	10 (83.3)	2 (16.7)	
Wound	4 (100)	0 (0)	
Peritoneal fluid	1 (100)	0 (0)	

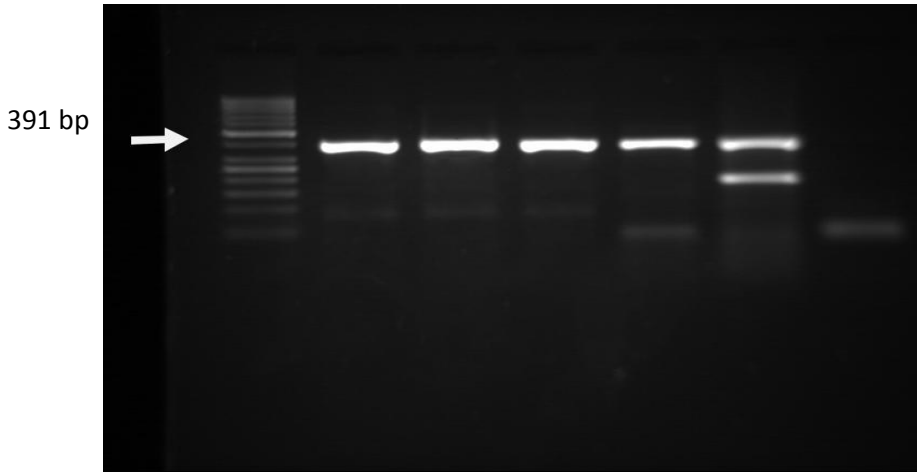

Figure 2. Gel electrophoresis of multiplex PCR products following amplification with specific primers for *blaVIM* gene (391bp). Lanes: 2: the 50bp Ladder; 3-6, clinical isolates for *blaVIM* gene,7; *blaVIM* gene *blaIMP* (232bp) positive control, 8; negative control.

Discussion

MBLs have been recognized in clinical isolates throughout the world. These strains are responsible for extended nosocomial outbreaks and accompanied by severe infections (19). In recent decade the carbapenem resistant *Pseudomonas aeruginosa* (CRPA) has emerged as an important pathogen. In similar study that was performed in the USA, the resistant rate ranged from 7.4% to 35.4% (20).

According to the results of another study in Taiwan 18.1% of all *P. aeruginosa* isolates in the ICUs of medical centers were CRPA. In the present study, the prevalence of *blaVIM* gene was assessed 14.6%. These different results implied the prevalence of CRPA was varied by geographic areas, specimen source, patient age, clinical setting and broad spectrum antibiotics consumption (4). Previous researches exhibited that IMP and VIM types of MBLs are also prevalent in Asian countries (21). VIM type is also the most frequent enzyme in Turkey (22).In a previous study in Iran, like our findings, *blaVIM* gene was detected among the isolates by PCR. MBL genes are placed on mobile genetic cassettes with other resistance determinants inserted into integrons, the isolates of *P. aeruginosa* producing these enzymes often show resistance to additional classes of drugs and act as multi-resistant. Our data also demonstrated that all of the VIM producing isolates are resistant to aminoglycosides and to fluoroquinolones that is in accordance with results of the similar study that was performed by Shahcheraghi in Tehran (21). Prevalence of VIM-type MBL producing *P. aeruginosa* strains in the recent similar study that was performed in Zanjan, Iran was 56% that also significantly was different (P<0. 05) from other districts of Iran and also higher than our results(14.6%). Most of MBL-producing isolates carried class 1 integron gene, which can easily spread the resistance encoding genes among these isolates. Several studies have also reported different frequencies of MBL positive isolates carrying class 1 integrons (2, 23). Other carbapenemase enzymes such as *blaDIM*, *blaSME*, *blaAIM*, *blaNMC*, *blaCcrA* are comparably infrequent and rarely in both their geographic occurrence and in the strains that they can be detected (10).

The *blaSME* genes are assumed to be chromosomal and non-mobile. The SME-1 β lactamase, along with the nearly identical SME-2 and SME-3, has been found sporadically in different geographical locations like London and United States (24). Infections caused by SME were small clusters of up to 19 isolates. (25). In this study all isolates were negative for *blaSME* or *blaAIM* genes by PCR screening that is in accordance with another similar study in 2009 (1). Every organization should usually check the resistant rate of pathogens and the antibiotics consumption, and report the information to infection control programs (26). The prevalence of antibiotic resistant to imipenem, meropenem and ceftazidime in our *P. aeruginosa* isolates were 97.9%, 97.9% and 79.2% respectively that showed higher resistance in comparison to other regions of our country such as Tehran, Ahvaz and Hamedan (8, 13). Several studies from other countries also showed the similar finding in recent years (27). Some researchers reported emergence of colistin resistant organisms in their study. Our results showed that about 50% of isolates were resistant to colisitin therefore selection of antibiotics to treat such patients should be carried out. In our study 36 (75%) of isolates were multidrug resistant but we couldn't find any significance relation between the MDR positive and demographic characteristics such as age, sex and source of infection. Several studies have found CRPA were more resistant to multiple drugs than carbapenem susceptible *P. aeruginosa* isolates and higher mortality rates of *P. aeruginosa* infection to be related to the site of infection, age, multidrug resistance, and improperness of empirical treatment (4). Phenotypic tests such as the Modified Hodge test (MHT) can be used to identify strains that need molecular testing in order to reducing the costs. The manufacturers declare that the test can also be performed for direct detection in clinical specimens (15).

In present study, carbapenemase gene was detected only in colonies because several studies have showed that EDTA can give false-positive results in some resistant *P. aeruginosa* isolates due to altered OprD levels and PCR confirmation is an important step (19). Out of 7 MHT positive cases for VIM in our study all of them were declared positive by PCR technique. Result of a study was carried out in 2007 to estimate different laboratory tests for identification of MBLs in Enterobacteriacae showed that MBL test detected 98% cases keeping PCR as the gold standard while only 0.03% was recognized as false positive (28). Our study has several limitations. First of all, this was a retrospective cross sectional study and we couldn't investigate many independent factors. Secondly, although we detected the presence of blaVIM gene, but we couldn't recognize AIM and SME genes because of the small sample size. Further studies should be performed to clarify if the presence of carbapenem-resistance genes affects the clinical outcome (4). In general, the multiplex PCR investigated in this study showed excellent performance and could supplement phenotypic tests in carbapenemase detection. The VIM prevalence in the present study was relatively high and this MBL enzyme could emerge among clinical isolates of *P. aeruginosa*. ICU patients because of broad spectrum antibiotic therapy and the presence of invasive devices are at increased risk for carbapenem resistance. As patients with infections caused by MBL-producing bacteria are at an intensified risk of treatment failure, fast determination of these organisms is necessary.

Conclusion

Because of high resistant rate of imipenem or meropenem so, it is suggested that a simple test based on CLSI recommendation such as MHT should be adopted to confirm MBL producing bacteria in clinical laboratories.

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Conflict of interest

None declared.

Financial disclosure

The authors declare that there is no conflict of interests to publish this article.

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