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Research Article

Study of the prevalence of Metallo-β-lactamase genes - bla VIM, bla IMP-1, and blaSPM of isolates of *pseudomonas aeruginosa* strains resistant to imipenem isolated from patients of Ahvaz hospitals

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ABSTRACT:

Introduction: *Pseudomonas aeruginosa* is one of the most important causes of nosocomial infections and has an inherent resistance to common antibiotics. Several antibiotics, including beta-lactams, are used to treat severe infections caused by this microorganism. The prevalence of nosocomial β-lactam antibiotic-resistant strains has been reported which Beta-lactamase enzymes are the most important factors involved in these resistance. Therefore, the aim of this study is to investigate the prevalence of Metallo-β-lactamase genes- bla VIM, bla IMP-1, and bla SPM of *p. aeruginosa* strains resistant to imipenem isolated from patients of Ahwaz hospitals.

Material and method: During a one-year period in 2016, 110 *p. aeruginosa* strains were collected from hospitals of Ahvaz. Antibiotic susceptibility of strains was determined by disk diffusion method in comparison to 6 common antibiotics in the region. The production of Metallo-β-lactamase in Imipenem resistant strains was investigated by hybrid disc method. Molecular analysis was performed by PCR method to detect blaVIM, bla IMP-1 and bla SPM genes. SPSS software was used to evaluate the relationship between variables.

Results: Agar disc diffusion results showed that among 110 strains, 67 [60.90%] strains were resistant to imipenem antibiotic. Resistance to other antibiotics was as follows:32 [29.09%] strains Meropenem resistant, 25 [22.72] strains Piperacillin resistant, 83 [75.45] strains Ticarsillin resistant, 87 [79.09] strains Ceftazidime resistant and 101 [91.81] strains cefepime resistant. Subsequently, by performing a phenotypic test for Metallo-β-lactamase production [MBL], it was found that 67 strains were metallo- β-lactam positive. The molecular studies of the PCR method were performed along with presence of the positive strains of bla IMP-1, bla VIM and bla SPM genes. 26.86% of the studied strains had the VIM gene, 43.13% had SPM gene and only 2.98% had the SPM-1 gene.

Conclusion: Based on the results, Imipenem resistance in Ahwaz is the same as all parts of the world, and many of these resistant strains have Metallo-β-lactamase genes. Regarding to the ability to transfer these genes to other strains, it is necessary to modify common methods, and to use better treatment protocols.

Keywords: *p. aeruginosa*, Antibiotic resistance, Metallo-β-lactamase, Imipenem

INTRODUCTION:

p. aeruginosa is an opportunistic pathogen, which is found in patients with impaired immune system and is the leading cause of nosocomial infections after Escherichia coli and *Staphylococcus aureus* [1] and the dominant pathogens of cystic fibrosis, chronic pulmonary obstruction And severe burns and patients admitted to the ICU [2, 3]. Also, organ transplant recipients are susceptible to fatal infections caused by this organism [3 articles].

This organism, especially at the hospital environment, is widely distributed and is considered as one of the most important causes of nosocomial infections [4]. Aside from Inherent drug resistance, the presence of multiple acquired drug resistance simultaneously has several serious drug-associated infections [5]. β-lactam enzymes are one of the most important causes of resistance to β-lactam and its coding gene is on the plasmid or on the chromosome of the bacterium. Many enzymes have been identified so far and are divided to several groups based on the substrate function and sensitivity to the inhibitor and genetic status.

These enzymes are divided into 3 groups based on the effect spectrum on antibiotics: 1- β-lactam with a limited range of effects that causes penicillin hydrolysis and cefoprazines. 2. Large-scale β-lactamase that causes hydrolysis of monobactams and cephems. 3. metallo- β-lactam, which affects all anti-*pseudomonasas*, except for manobactams [6, 7]. Due to the complexity of these enzymes, four important groups were proposed by Richmond Sykes *et al*, [Ambler, Inoue, Mitsuhashi, and Bush] among them the Ambler classification is more acceptable. Ambler classification secretes the beta-lactamase enzymes into 4 A-D classes based on the sequence of the primary amino acid. Each of the four groups is present in *Pseudomonas*. Type A, C, and D are serine β-lactamase, whereas type B is metallo-β-lactamase. ESBLs are class A beta-lactamases that have the ability to hydrolyze third-generation cephalosporins and monobactams. But they are not able to hydrolyze carbapenems. Most of these enzymes are derivatives of SHV and TEM β-lactam. TEM1 and its derivatives are reported more often in *Enterobacteriaceae* and SHV-derived β-lactam are reported more often in *Klebsiella pneumoniae*, while type PER and VEB ESBLs have been found to be more frequent in *P. aeruginosa* in addition to *Enterobacteriaceae* [8,9,10,11].

PER and VEB are more chromosomal in *pseudomonas*. Type PER has less similarity to SHV and TEM and is detected in Pseudomonas for the first time, and PER1 only has 18-20% similar amino acid to TEM and SHV and is able to hydrolyze penicillins and cephalosporins and its activity is also inhibited by clavonic acid. The PER1 enzyme has been found both on the chromosome and on the plasmid, since the enzyme has been reported in several bacteria, so its gene is located on the transportable elements. This enzyme has high resistance to ceftazidime, cefotaxime and aztreonam [12, 13, 14, 15].

The presence of some broad-spectrum β-lactam on transposons and the possibility of their movement on the genome may also prompt expression of resistance genes and the emergence of new resistance genes to other antibiotics of broad-spectrum cephalosporins [such as the GES-1 gene] and even Carbapenems. Therefore, appropriate policies should be adopted not only for the use of broad-spectrum cephalosporins, but also for non-beta-lactam antibiotics. Metallo-β-lactamase in Class B of the Ambler classification and Group 3 of Bush *et.al*, classify need bivalent cations such as zinc metal as a cofactor for their enzymatic activity, are coded by chromosomes or plasmids, and have a wide spectrum substrates so they hydrolyze beta-lactams, except for manobactam [aztreonam]. The molecular enzymes are divided into four groups, based on the molecular structure, called GIM, SPM, VIM and IMP. IMP1 is the first Metallo-β-lactamase described in *Pseudomonas*, its gene is bla IMP, which its presence in *pseudomonas* and other gram-negative bacillus, was reported in Japan. The blaVIM was the dominant gene of imipenem resistant *pseudomonas* strains isolated from clinical specimens [16, 17, 18, 19]. Metallo-β-lactamase has been identified from clinical isolates around the world with an increasing incidence over the past years, and the strains produced by these enzymes are responsible for long-term hospital infections with serious consequences. A Metallo-β-lactamase isolate at the hospital environment causes problems in treating patients. For this reason, it is a serious concern for the person who controls the infection, especially in burn units [21, 20, 22]. Therefore, the present study was carried out to investigate the prevalence of Metallo-β-lactamase genes-bla IMP-1, bla VIM, and

blaSPM in isolates of *p. aeruginosa* imipenem resistant strains isolated from patients of Ahwaz hospitals.

MATERIALS AND METHODS

In this descriptive cross-sectional study, 110 clinical samples were isolated to study the resistance genes of Metallo-β-lactamase in *P. aeruginosa* strains [isolated from burns] during one year. Then the specimens were cultured in a nutrient-agar culture medium and incubated at 37 ° C for 24 hours. Then, *p. aeruginosa* was detected by biochemical tests such as catalase, oxidase, TSI, Citrate, Andel, MRVP and pigment production and Saved at 4 ° C after it was cultured in liquid LB culture medium and glycerol was added [final concentration 20%].

Antibiotics on the disk are distributed over the Muller Hinton Agar medium according to the diffusion law from the concentrated point to the diluted point. Therefore, the antibiotic concentration produced on the medium because the bacteria grow on the basis of sensitivity or resistance to the antibiotic concentration around the disk and forms an inhibition zone. Antibiotic susceptibility test was performed by disk diffusion [Kirby-Baer] based on CLSI standards, using antibiotic disks imipenem [10µg], ceftazidime [30µg], ticarsillin [30µg], ciprofloxacin [5µg], cefepime [30µg], piperacillin [100µg]. That way First, a suspension was prepared at a concentration of 0.5 McFarland, and after full suspension distribution on the Muller Hinton Agar, the disks were placed on the medium at a distance of 2 cm from each other, and after 16-18 hours incubation in The temperature of 37 ° C, the diameter of the lack of growth halo around each disc was measured and its results were recorded. According to the table with the disks, we reported the antibiotic test report for each antibiotic as sensitive, semi-sensitive and resistant. The strain of *p. aeruginosa* [ATTCC 27853] was used as control strain [23, 24, 25, 26].

Determination of metallo-β-lactam gene:

To extract bacterial DNA [chromosome and plasmid], simple boiling method [BOILING] was used. First 2 to 3 colonies were taken from the culture, solved in 500µ l distilled water into micro-tubes of eppendorf and after the vortex of the solution, it was obtained for 10 minutes in a boiling Ben Murray and at the end micro-tubes were centrifuged for a period of 10 minutes at 12000 g round and the supernatant was used for PCR test [].In order to reproduce the extracted DNA using the PCR reaction, the composition of the main mixture with a volume of 25 µL was as follows:

2.5 µl PCR buffer, 0.625 µl dNTP mixture, 0.75 µl of 50 mM magnesium chloride, 0.4 µl Taq polymerase enzyme, 0.5 microliter of each primer, DNA template 5 µl and 14.73 µl of distilled water. The PCR program was given to thermocyclers for 33 duplication cycles, which included the following steps.

The initial step of separating or opening of the two strands for 5 minutes at 94 ° C, the opening phase of the two strands for 1 minute at 94 ° C, the step of connecting of the primers for 1 minute at 54 ° C, and the elongation stage Or the target string production for 1.5 minutes at 72 ° C. PCR products were examined by electrophoresis with 1.5% gel in TBE buffer. The gels were stained with ethidium bromide. Then, PCR products were observed with UV light. The primers used in this study are presented in Table 1.

Table 1: Primers used in this study

Primer Name	Sequences 5'→3'
VIM F	GTTTGGTCGCATATCGCAAC
VIM R	AATGCGCAGCACCAGGATAG
SPM F	CTGCTTGGATTCATGGGCGC
SPM R	CCTTTTCCGCGACCTTGATC
IMP-1 F	GAAGGCGTTTATGTTCATAC
IMP-1 R	GTAAGTTTCAAGAGTGATGC

RESULTS

Results for drug resistance:

After isolating and recognizing 110 *p. aeruginosa* strains from clinical specimen, resistance pattern of these strains to antibiotics of Ceftazidime [CAZ], imipenem [IMI], piperacillin [PRL], meropenem [MEM] ,

cefepime [CPM] and ticarsillin [TI] was studied by disk diffusion according to the clinical laboratory standard scales of CLSI [chart 2-3]
Study of antibiotic resistance pattern of isolated *p. aeruginosa* strains showed that highest level of antibiotic resistance is referred to cefepime and the least resistance is related to piperacillin [table3-3][chart 2-3].

Table 3-3 rate and frequency percentage of strains resistant to used antibiotics

Antibiotic	Antibiotic	تعداد نمونه مقاوم
Imipenem (10µg)	٪60/90	67
Ceftazidime (30µg)	٪79/09	87
Meropenem (10µg)	٪29/09	32
Cefepime(30µg)	٪91/81	101
Ticarcillin(75µg)	٪75/45	83
Piperacillin (100µg)	٪22/72	25

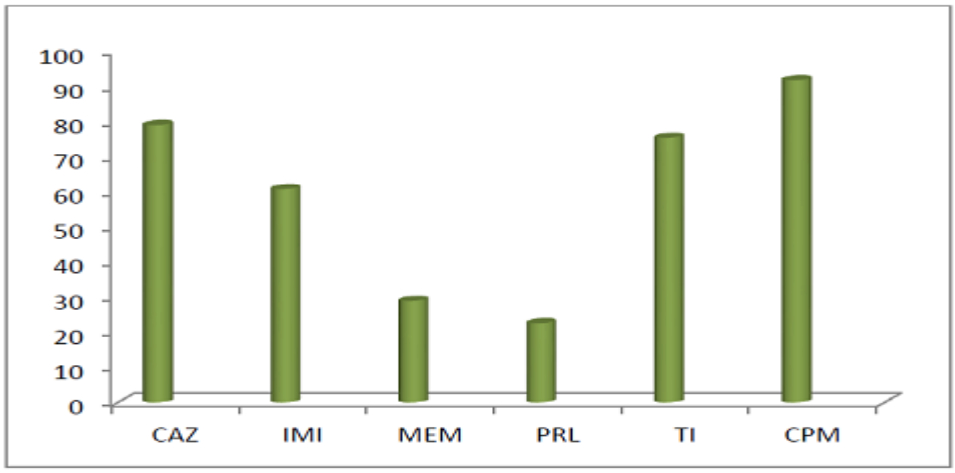

Chart 2-3 frequency percentages of strains resistant to used antibiotics

Results for DNA and PCR isolation

After DNA extraction from isolates of *p. aeruginosa* isolated from clinical specimen by boiling, in order to study the extracted DNA qualitatively, DNA electrophoresis on agarose 0.8 was performed. Figure 1-3 shows the DNA electrophoresis results.

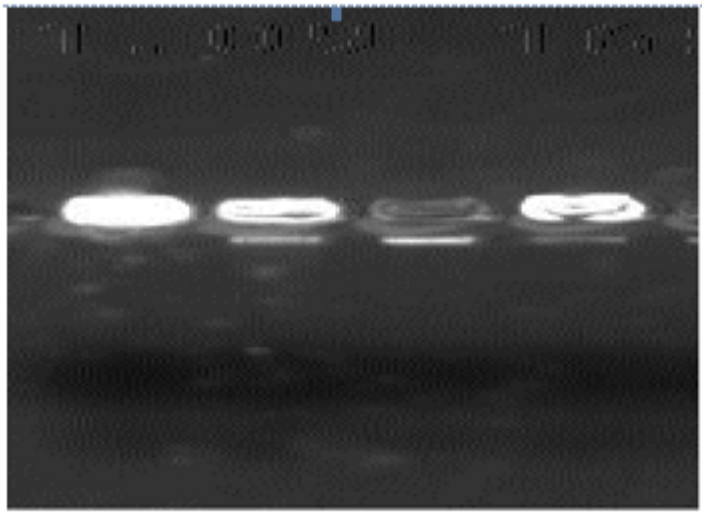

Figure 2-3 electrophoresis of extracted DNA of some isolates of *p. aeruginosa* isolated from clinical specimen

Results of VIM

After DNA was extracted from imipenem resistant strains PCR was done for VIM gene. Based on designed primers for this gene, it is expected that a 382-bp band will be produced in the PCR reaction due to replication of this gene. The results of the PCR reaction using the VIM primer on isolated clinical specimens showed that among 67 samples of *p. aeruginosa*, 18 specimens had this gene and produced 382 bp band in the PCR reaction [Fig. 21.3]

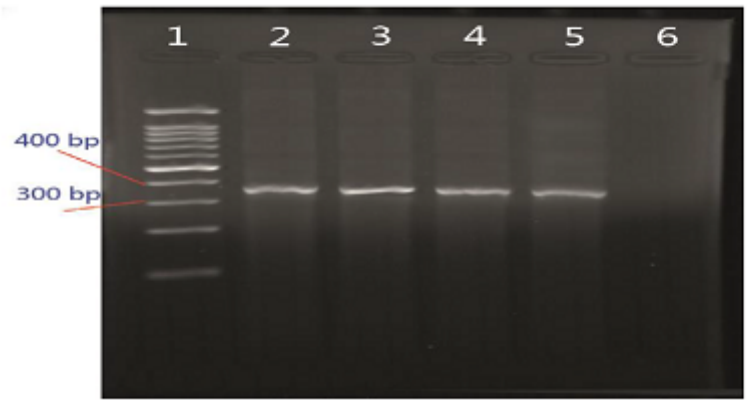

Figure 21-3 PCR results of vim gene

The results of the identification of imipenem resistance gene

After DNA extraction of imipenem antibiotic resistant strains PCR was performed for SPM gene. Based on designed primers for this gene, it is expected that the replication of this gene will produce 771 bp bands in the PCR reaction. The results of the PCR reaction using the SPM primer on isolated clinical specimens showed that among the 67 samples of *p. aeruginosa*, 9 specimens were eligible for this gene and produced 771 bp band in the PCR reaction [Fig. 22-3]

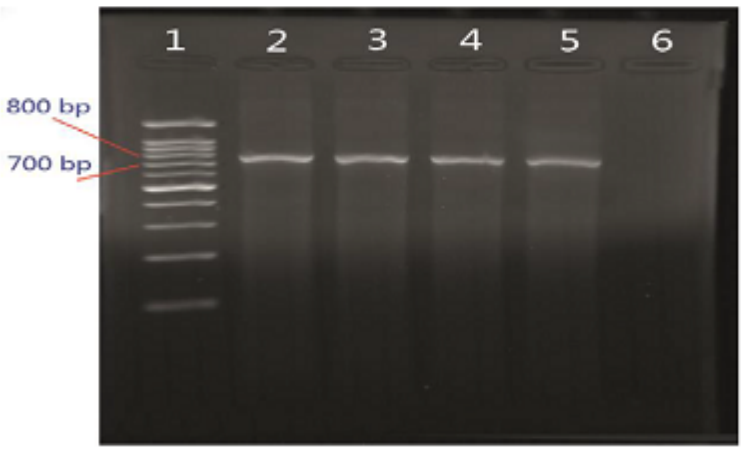

Figure 21-3 PCR results of SPM gene

Column 1 Marker, column 2 Positive control of *p. aeruginosa* ATCC 27853 and Columns 3, 4 and 5 *p. aeruginosa* isolated from clinical specimens with SPM gene and column 6 negative control.

Results of IMP1

After DNA extraction of imipenem antibiotic resistant strains PCR was performed for IMP1 gene. Based on designed primers for this gene, it is expected that the replication of this gene will produce 587 bp band in the PCR reaction. The results of the PCR reaction using the IMP1 primer on isolated clinical specimens showed that among the 67 samples of *p. aeruginosa*, 2 specimens were eligible for this gene and produced 587 bp band in the PCR reaction [Fig. 23-3].

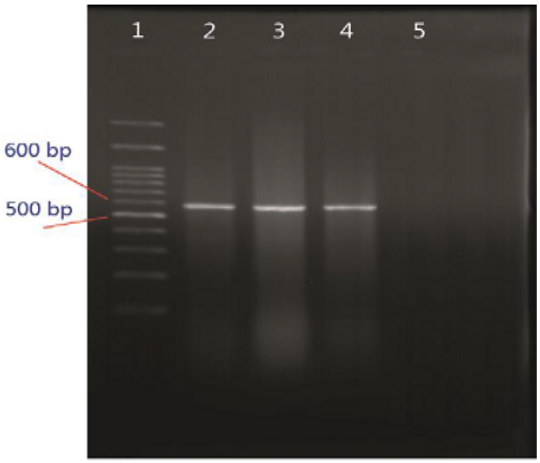

Figure 23-3 PCR results of IMP1 gene

DISCUSSION:

p. aeruginosa strains producing metallo- β-lactam are a serious clinical threat that has caused concern to physicians who treat infections caused by these bacteria. IMP is the first Metallo-β-lactamase known in *p. aeruginosa*. The potential risk of a rapid spread of IMP to other bacterial species has been of great concern. Metallo-β-lactamase have been identified from clinical isolates in different parts of the world with an increasing incidence over the past years, and the strains producing these enzymes are responsible for long-term hospital infections with serious consequences [27 and 28] . An isolate producing Metallo-β-lactamase at the hospital environment causes problems in treating patients and therefore a serious concern for a person controlling the infection, especially in the burn unit. In a study in Japan, it has been shown that simplified infected patients with *p. aeruginosa* producing Metallo-β-lactamase receive various antibiotics for the treatment, also the deaths caused by the infection by these bacteria occurs more than those caused by *p. aeruginosa* Negative metallo-β-lactamase [29]. Due to the fact that *p. aeruginosa* is an opportunistic pathogen in immunocompromised patients and it is a major contributor to hospital and burn infections, the accurate determination and rapid reporting of such enzymes, both epidemiologically and in order Helping the doctor choose appropriate antibiotics for successful treatment of patients, controlling the spread of multi-drug resistant isolates, and preventing the spread of such infections in hospitals, will be worthwhile with the increasing prevalence of gram-negative bacilli producing Metallo-β-lactamase in many countries, simple and accurate tests to identify strains producing these enzymes are required. Disc diffusion agar results showed that among 110 strains, 67 [60.9%] strains were resistant to imipenem antibiotic. Resistance to other antibiotics was as follows:

32 [29.09%] strains of Meropenem resistant, 25 [22.72] resistant to piperacillin, 83 [75.45%] strains of Ticarsillin resistant, 87 [79.09%] strains resistant to ceftazidime and 101 [91.81%] Strain resistant to cefepime. Subsequently, by making a phenotypic test for Metallo-β-lactamase production [MBL], it was found that 67 strains were Metallo-β-lactamase positive. The molecular studies of the PCR method were performed with the presence of the positive strains of bla IMP-1, bla VIM and bla SPM genes. 26.86% of the studied strains had the VIM gene, 43.13% had SPM gene and only 2.98% The SPM-1 gene was.

In a study by Park and et al, 2003, among 99 *p. aeruginosa* isolates resistant to Imipenem, 31 isolates were positive of Metallo-β-lactamase by phenotypic methods, while PCR was used to isolate 29 isolates of the VIM gene and 2 other isolates of the IMP gene. The researchers also reported that VIM is a major Metallo-β-lactamase in *p. aeruginosa* in Korean hospitals [30]. In a study by Luzzaro et al in 2004 in Italy, the researchers showed that among 82 strains of *p. aeruginosa* resistant to imipenem four strains were were Metallo-β-lactamase positive by PCR and Etest, and the PCR results revealed that in all 4 strains, the vim gene was present. The researchers argued that *P. aeruginosa* strains producing Metallo-β-lactamase were important cases of imipenem resistant strains among isolated strains of patients [31]. In another study by Kim et al. during 2005, among 116 isolates of *p. aeruginosa* resistant to imipenem, 22 isolates were Metallo-β-lactamase positive by E test, whereas by PCR, 21 isolates of the VIM gene were present and a remaining isolate they were not classified in molecular formations [32].

CONCLUSION:

All microbiological tests should be able to detect *p. aeruginosa* strains that produce MBL from strains that use other mechanisms to resist carbapenems. Identification and initial determination of *p. aeruginosa* MBL producer may prevent the spread of multi-drug resistant strains. Based on the results, Imipenem resistance in Ahwaz is the same as in all parts of the world, and many of these resistant strains have Metallo-β-lactamase genes. Regarding to the ability to transfer of these genes to other strains, it is necessary to modify common methods, in addition to use better treatment protocols.

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