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Research Article

Detection of *bla* $_{NDM\text{-}1}$, *bla* $_{VIM}$, and *bla* $_{IMP}$ genes in multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* from clinical isolates in Tehran hospitals

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ABSTRACT

Acinetobacter baumannii and *Pseudomonas aeruginosa* are non-fermentative Gram-negative bacilli with the ability to produce β-lactamase, they are often resistant to β-lactam drugs which cause serious infections.

The aim of this study was to investigate the prevalence of metallo β-lactamase (*MBL*) genes (*bla* $_{NDM\text{-}1}$, *bla* $_{VIM}$, and *bla*$_{IMP}$) among isolated multidrug-resistant *A. baumannii* and *P. aeruginosa*.

A total of 212 imipenem and meropenem resistant *A. baumannii* (107) and *P. aeruginosa* (105)were included in the study. Antimicrobial susceptibility was determined by the disk diffusion agar method. Initial screening for MBL enzyme production was performed by the combined disk diffusion test phenotypic (CDDT) andMICmethods . Polymerase chain reaction (PCR) was performed for the detection of MBL (*bla* $_{NDM\text{-}1}$, *bla*$_{VIM}$, and *bla* $_{IMP}$) genes and gene sequencing was performed for representative isolates. Of 107 imipenem and meropenem resistant *A. baumannii*, 99 (92.5%) were *MBL* enzymes produced.

After conducting PCR, 64 cases (60.4%) were *VIM* positive. Of 105 imipenem and meropenem resistant *P.aeruginosa*, 93 (88.57%) were producers of *MBL* enzymes. After conducting PCR, 69 (65.7%) were *VIM* positive and 36 (34.3%) were *IMP* positive. Furthermore, *bla*$_{NDM\text{-}1}$ was not observed in isolated *A. baumannii* and *P. aeruginosa*.

These data suggest that among genes encoding β-lactamase, the prevalence of *bla-*$_{IMP}$ in *P. aeruginosa* shows an increase in comparison with other countries. Furthermore, patients with bacteria carrying MBL cannot be treated easily, and therefore, increased research and resources in optimizing treatment, infection control, and antibiotic stewardship are required.

Keywords: *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *MBL genes*, PCR

INTRODUCTION:

The indiscriminate prescription of antibiotics in the community for hospital-acquired infections is considered as a possible mechanism for the spread of infection [12]. The dissemination ofantibiotic resistant genes between bacterial species in infectious diseases is a serious and worrying

problem [23].Carbapenems, for example, are a type ofβlactam drug, which are often used as a last resort for the treatment of infections caused by drug resistant Gram-negative bacteria [7]. The emergence and spread of multidrug-resistant *P. aeruginosa* and *A. baumannii*and their genetic potential to carry and transfer antibiotic resistance poses a major threat in hospitals [18].

Resistance to carbapenem is often caused by various mechanisms, includingthe production ofmetallo β-lactamases (*MBL*)bymicroorganisms. According to the sequence of amino acids, lactamase enzymes belong to the Ambler class B Group classification and the Bush classification (based on the type of substrate) [22]. Mostmetallo-β-lactamasegenes(such as*VIM*, *IMP*, and NDM1) are found as a gene-depending cluster on class *Iintegrons*[8].VIMs, for instance, have been shown in multidrug resistant(MDR) *P.aeruginosa* in Italy, while more than 33 types of *VIM* have been reported worldwide. *IMP* enzymes, on the other hand, were first identified in Japan andcurrently more than 20 different allotypes of *IMP* have been described. New Delhi *Metallo-beta-lactamase*-1 (*NDM-1*) is another new type of *MBL* that was first detected in a *Klebsiella pneumoniae* and *Escherichia coli* isolate from a Swedish patient of Indian origin in 2008[9].

The aim of this study is to determine the prevalence of *bla* $_{NDM\text{-}1}$, *bla*$_{\text{VIM}}$, and*bla*$_{\text{IMP}}$genes among *A.baumannii* and *P.aeruginosa* resistance to imipenem and meropenem isolated from patients in three hospitals in Tehran.

MATERIAL & METHODSA

Collection of resistant *P.aeruginosa* and*A.baumannii* isolates were recovered from patients who were admitted to Milad, Motahari, and Taleghani Hospitals in Tehran during October 2012 till June 2013.Samples were transferred rapidly under standard conditions to the Mofid hospital research central laboratory. Following this, all conventional biochemical methods and differential tests used for re-examination and phonotypical detection of isolated bacteria were according to the following steps.

Antimicrobial Susceptibility Test (AST):

The antimicrobial susceptibility test of the isolated organisms were completed by the disc diffusion method using the Kirby–Bauer technique [3]. According to the recommendation of CLSI [6], all tests were performed on Mueller Hinton agar. The surface was lightly and uniformly inoculated by a cotton swab. Prior to inoculation, the swab stick was dipped into a bacterial suspension having visually equivalent turbidity to 0.5 McFarland standards. The swab stick was then taken out and squeezed on the wall of the test tube to discard extra suspension. Inoculated plates were incubated at 35°C for 24 h. On the next day, plates were read by taking measurements of zone of inhibition. Results were recorded and graded as resistant (R), intermediate (I) and sensitive (S), according to the reference zone of inhibition of particular antibiotic.

Determination of MIC by Broth Microdilution Method:

The microdilution method was used to determine the minimum inhibitory concentration (MIC) of an antimicrobial agent. The results were interpreted according to the recommendation chart of CLSI(6).

Phenotypic Detection of *MBL*s

The combined disk diffusion test (CDDT) was performed for the identification of MBLs by imipenem and meropenem (Mast Group, Merseyside, UK) alone and in combination with EDTA.First, 0.5 molar EDTA solution was prepared and sterilized by autoclave, and imipenemand meropenem resistant strains were inoculated on Mueller Hinton plates.A 10 mcg imipenem disc and a disk containing 750 mg imipenem equivalent to 10 micrograms (EDTA) were placed on the surface of the plates with a suitable distance. Plates were then incubated at 35°C for 24 h.An increase in zone diameter ≥7 mm around the imipenem+EDTA and meropenem+EDTA disks compared to that of imipenem and meropenem disks alone,

respectively, were considered positive for MBL production [9].

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) test was carried out by the E-test method for imipenem-resistant strains. Mueller-Hinton agar plates, the recommended medium, were streaked by using cotton swabs. The E-test strips were impregnated with imipenem and then is placed on an agar surface, which were incubated at 35°C in air for 16 to 20 hours. *P. aeruginosa* strains with MIC ≥8 µg/ml to imipenem are referred to as resistant (6).

DNA Extraction and PCR

The whole genomic DNA from cultured strains were prepared using the boiling method. The brewing kit, Accu power premix (Bioneer Co., Korea), was used for PCR products. The *bla* $_{NDM-1}$, *bla* $_{VIM}$, and *bla* $_{IMP}$genes were detected by the PCR method using specific primers as described by Fallah et al [9]listed in Table1.

Table1: Listed primer

Primer	Primer sequence (5′-3′)	Product size (bp)
Bla$_{NDM-1}$-F *Bla*$_{NDM-1}$-R	GGGCCGTATGAGTGATTGC GAAGCTGAGCACCGCATTAG	758
Bla$_{VIM}$-F *Bla*$_{VIM}$ -R	GTTTCGTCGCATATCGCAAC AATGCGCAGCACCAGGATAG	382
Bla$_{IMP}$-F *Bla*$_{IMP}$ -R	GAAGGCGTTTATGTTCATAC GTATGTTTCAAGAG TGATGC	588

The PCR mixtures (20 µl) contained 1 µl of DNA and 1.5µl of each primer, and the PCR master mix (Sinaclon, Iran) was diluted in distilled water. Thermal cycles were programmed by the Thermocycler instrument (Eppendorf Company). Reaction conditions for all primers were as follows: Initial denaturation at 95°C for 4 min, 35 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min; followed by a final extension step of 72°C for 5 min. PCR products were revealed by electrophoresis on a 1% agarose gel (Fermentase) using a 1× TBE buffer and a subsequent exposure to UV light in the presence of a KBC power load dye (GelRed Nucleic Acid Gel Stain, 10,000_ in water, Kawsar Biotech Co., Tehran, Iran).*P.aeruginosa* ATCC 27853 was used as control strain.

Sequencing Method: The PCR purification kit (Bioneer Co., Korea) was used to purify PCR products and sequencing was performed by the Bioneer Company (Korea). The nucleotide sequences were analyzed with Chromas 1.45 software and the BLAST program from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST).

RESULTS

In this study, 212 isolates of *A. baumannii* (n=107) and *P. aeruginosa* (n=105) resistant to imipenem and meropenem were collected from three hospitals in Tehran. 99 Of 107 (92.5%) MDR acinetobacters resistant to imipenem and meropenem, were producers of MBL enzymes by the CDDT phenotypic method. After conducting PCR, 64 cases (60.4%) were *VIM* positive but *IMP* and *NDM1*was not observed. A total of 93 of 105(88.57%) MDR pseudomonas resistant to imipenem and meropenem were producers of MBL enzymes by using the CDDT phenotypic method. After conducting PCR, 69 (65.7%) were *VIM*positive and 36 (34.3%) were IMP positive, and *bla* $_{NDM-1}$was not observed.

Antibiotics	Resistant (*A. baumannii*)n (%)	Resistant (*P. aeruginosa*)n (%)
Piperacillin	104(97.2%)	96(91.43%)
Ticarcillin	104(97.2%)	104(99.05%)
P-Tazobactam	103(96.27%)	89(84.76%)
Ceftazidime	102(95.33%)	91(86.67%)
Imipenem	107(100%)	105(100%)
Meropenem	107(100%)	105(100%)
Colistin	7(6.5%)	4(3.81%)
Gentamicin	55(51.4%)	71(67.62%)
Tobramycin	69(64.48%)	101(96.19%)
Amikacin	84(87.5%)	99(94.3%)
Ciprofloxacin	102(95.33)	102(97.4%)
Levofloxacin	100(93.46%)	102(97.4%)

Table 2: The comparison of antibiotic resistance of isolated *A. baumannii* and *P. aeruginosa*

Nucleotide sequencing for PCR products confirmed the presence of *bla* $_{IMP}$ and *bla* $_{VIM}$ genes in *P. aeruginosa* and *bla*$_{VIM}$genes in A. *baumannii*(Fig. 1,2).

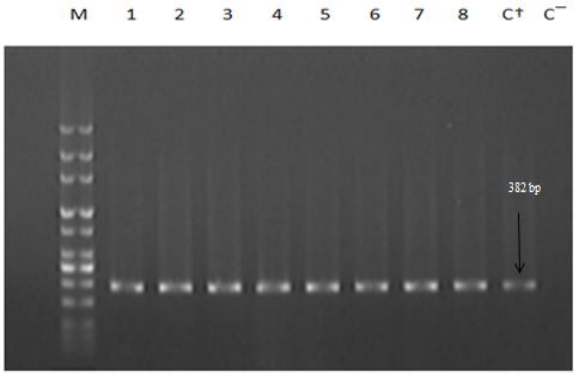
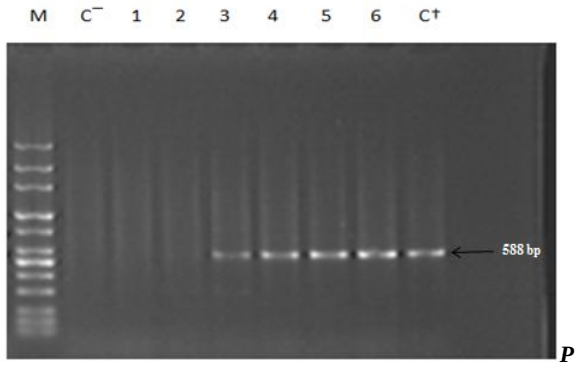


Figure 1 : PCR Amplification of *VIM* Gene in a Strain of A. baumannii

PCR Products Gel Electrophoresis for VIM GeneLanes: M: 100 bp Marker, C +: Positive Control, 1 to 8: PCR Product for *VIM* Gene,C -: Negative Control.

Figure 2. PCR Amplification of *IMP* Gene in a Strain of *Pseudomonas aeruginosa*



P CR Products Gel Electrophoresis for IMP Gene

Lanes: M: 100 bp Marker, C +: Positive Control, 3 to 6*: PCR Product for IMP Gene,C-:* ***Negative Control.***

The results for the resistance of *A. baumannii* isolates are as follows: imipenem, meropenem, andcefotaxim (100%);cotrimoxazel (98.13%);cefepime, piperacillin, andticarcillin (97.2%);*P. tazobactam*(96.27%);ciproploxacin andceftazidime (95.33%);lerofloxacin (93.46%);amikacin (87.5%);tobramycin (64.45%);tetracycline (61.68%);gentamicin (51.4%);and colistin (6.5%).The results for the resistancy of *aeruginosa* isolates are as follows: imipenem and meropenem (100%);ticarcelin and carbenicillin (99.05%);cirrokloxacin &levofloxacin (97.4%);norfloxacin andtobramycin (96.19%);amikacin (91.3%);aztreonam (92.38%);piperacillin (91.43%);cefazidime (86.67%);*P. tazobactom* (84.76%);gentamicin (67.62%);and colistin (3.81).For all samples, MBL test using a combination of the disk diffusion test method (CDDT) was performed and the results were as follows: 99(92.5%) cases of *A. baumannii* and 93 (88.57%) cases of *P.aeruginosa*, respectively, were MBL positive.12 antibiotic disks were common between *A. baumannii* and *P.aeruginosa* isolates. The comparison of antibiotic resistance for isolated bacteria is illustrated in table II.

The results of the PCR tests for *bla* $_{NDM-1}$, *bla*$_{VIM}$, and*bla*$_{IMP}$genes showed that the prevalence of the *bla*$_{VIM}$gene in *A. baumannii* and *P.aeruginosa* is 60.4% and 65.7%, respectively. In cases of the *bla* $_{IMP}$gene in *A. baumannii* and *P.aeruginosa*, however, it was 0% and 34.3%, respectively. The *bla* $_{NDM-1}$gene was not observed in both strains.

DISCUSSION:

Non-fermentative Gram-negative bacilli such as *A. baumannii* and *P.aeruginosa* are globally thought of as the main pathogens that causes severe invasive infections, particularly in ICU hospitalized patients [21, 24]. As well as this, this are commonly resistant to multiple drugs [16, 17]. The major cause of their resistance to β-lactam drugs such as cephalosporins, penicillins, andcarbapenems is due to the production of β-lactamase [11, 16, 17]. These organisms are usually resistant to all or most of the available drugs and are able to obtain resistance genes [4, 19, 24].In this study, all isolated *A. baumannii* and *P. aeruginosa*samples were resistant to meropenem and imipenem. Antibiogram by the disk diffusion agar method was used for all samples simultaneously. Following this, MIC of colistin was performed according to the CLSI

protocol in isolated A. baumannii.Due to the phenotypic detection of MBLs, MBL testsused a combination of the disc method (CDDT) and the results were as follows: 99(92.5%) cases of A. baumannii and 93 (88.57%) cases of P.aeruginosa were *MBL* positive. After conducting PCR for *bla* $_{NDM\text{-}1}$, *bla* $_{VIM}$, and*bla* $_{IMP}$genes, the results showed that the prevalence of the bla_{VIM} gene in A. baumannii and P.aeruginosa is respectively 60.4% and 65.7%, while in cases of blaIMPgenes in A. baumannii and P.aeruginosa, isolates were 0% and 34.3%, respectively. There are different genesthatencode the β-lactamases, among *MBL* genes, *IMP* is thought to be more important in Iran [9].According to these results, the prevalence ofbla_{IMP}in isolated P.aeruginosa is 34.4% which is noteworthy.In a study by Khosravi, performed on 90 cases of imipenem-resistant *P.aeruginosa*, 35.5% were *MBL* positive. They reported 56.2% were *VIM* positive and 43.7% *IMP* were positive in the *P.aeruginosa*[13]which resembles our results.Regarding the clinical significance of *A. baumannii* and P.aeruginosa in healthcare-associated infections in hospitals, we found a significant association between the *VIM* gene and these strains.This was due to the *bla VIM* being identified in both bacterial strains on a large-scale (60.4% in *A. baumannii* and 65.7% in *P.aeruginosa*isolates). In a study conducted by Peimani et al on 63 resistant *A.baumannii*to imipenem and meropenem, 49% (n=31) were *MBL* positive.Among them, 29% (n=9) *VIM*positive and 61% (n=19) IMP positive were reported [20]. Furthermore, in a study by Franco on negative bacilli isolated from blood cultures, 34% of the samples were resistant to imipenem. Among them, 30%were*MBL* positive and 19%were *VIM* positive. None were reported for *IMP*[10].These results are very different from our study.It seems that this inconsistency is due to the bacterial isolates included in the studies.Lagatolla reported that among 89 P.aeruginosa resistant to imipenem, 72% were *VIM* positive,which wassimilar to our research. However, there was no reported *IMP*. In reverse, we found 34% *IMP* positive P.aeruginosa[14]. In a study conducted by Fallah et al, 86 of the 108(86/8%) A. baumannii were MBL positive and the prevalence of *VIM* and *IMP* genes were 17.4% and 3.48%, respectively[9]. In our study, in the case of *VIM*, the percentage of positive A. baumanniiwas 43% higher.In a study conducted by Lee et al, among 387 *Pseudomonas* spp resistant to imipenem, 44 (4/11%) were MBL positive and100% were *VIM*positive, while no *IMP* positive caseswere reported. From 38 cases of 267 *Acinetobacters* spp, 2/14% were MBL positive, while 71% (n=27) and 28.9% were *VIM* and *IMP* positive, respectively[15]. These results are in contrastto our findings. Amudhan et al also reported that among 179 isolated Acinetobacter and Pseudomonas spp, 144 cases (80.4%) were *MBL* positive, 89 were *VIM* and 2 were IMP positive. One alsohad both genes [2]. These results substantially resemble to our findings and both studies were performed within the same year. Similarly, In Aksoy et al's study, 96% of 50 cases of A. baumannii were MBL positive [1]. Chen et al reported that in 11,298 samples consisting of P.aeruginosa, *A. baumannii*, *K. pneumonia*, and*Escherichia coli* only four $bla_{NDM\text{-}1}$genes (0.03%) were reported[5], while in in the present study no positive cases were found.As it can be observed, resistance patterns in different countries are variable. It appears that the pattern of resistance over different years and even the results of geographically different countries are effective factors in these variations.Hence, the time of the study and the geographical area may be considered as a contributing factor in the cause of these differences. As well as this,studymethods and typeof antibioticdisksused affect the results. For example, meropenem or imipenem disk usage in the diagnosis of *MBLs* can show minor differences[7].

CONCLUSIONS:

The emergence of resistance due to *MBLs* is one of the main concerns in our country and bacterial resistance to antibiotics is substantially observed

at this present time. Furthermore, patients with bacteria carrying *MBL* cannot be treated easily and because the genes producing these enzymes such as *IMP*, *VIM*, and *NDM-1* are on moving genetic elements such as plasmids, transposons, and integrons, these elements can be easily transferred among bacteria of the same species and even from a bacterium to another bacterium. Hence, we should consider isolating these patients to avoid indiscriminate use of antibiotics and also reduce the costs of long-term treatment. The results demonstrated that among genes encoding β-lactamase, the prevalence of *bla* $_{IMP}$in *P. aeruginosa*showed an increase in comparison with other countries. *bla*$_{NDM-1}$was also not observed in isolated*A. baumannii* or P.aeruginosa.

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