Detection of Metallo-β-Lactamase Producing *Pseudomonas aeruginosa* Isolated from Public and Private Hospitals in Baghdad, Iraq

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Abstract- Metallo-β-lactamase (MBL) producing *Pseudomonas aeruginosa* has been reported to be an important nosocomial infection. Its intrinsic and acquired resistance to various antimicrobial agents and its ability to develop multidrug resistance imposes a serious therapeutic problem. Different clinical samples were collected from public and private hospitals in Baghdad city, Iraq. Bacterial identification was done using conventional cultural, biochemical tests, and VITEk 2 system. Minimum inhibitory concentration (MIC) testing was performed using VITEK 2 automated system. Each *P. aeruginosa* isolates showed resistance to Carbapenems (Imipenem and Meropenem) were subjected to Imipenem-EDTA combined disc synergy test (CDST) to investigate the production of MBL (confirmative test). The presence of bla-genes encoded IMP, VIM, and SPM-1 was detected by conventional PCR technique. A total of 75 *P. aeruginosa* isolates were isolated, 16 (21.3%) were able to grow on MacConkey agar supplemented with Meropenem 4mg/L (MMAC). The MIC of different antibiotics showed that 6 (37.5 %) isolates were Carbapenem resistant, MIC ≥16 µg/ml while 4 (25%) isolates appear to be MBL producer using CDST test. PCR assay revealed that 3 (50%), 1 (16.6%) of the carbapenem resistant isolates harbored blaIMP, blaSPM-1 genes, respectively. blaVIM gene was not detected in this study. The prevalence of multi-drug resistant *P. aeruginosa* isolates especially Carbapenem resistant bacteria was increased in Baghdad province. The blaIMP was the predominant among the MBLs genes in *P. aeruginosa* in this study.

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Keywords: *Pseudomonas aeruginosa*; MBL; Carbapenems; ESBL; CRPA; Bla genes

Introduction

Pseudomonas aeruginosa producing MBLs were first reported from Japan in 1991 and since then has been described from various parts of the world including Asia, Europe, Australia, South America, and North America (1). MBLs belong to Ambler class B and have the ability to hydrolyze a wide variety of β-lactam agents, such as penicillins, cephalosporins, and carbapenems and consist of five groups of enzymes, namely IMP, VIM, SPM, GIM and SIM These enzymes require zinc for their catalytic activity and are inhibited by metal chelators, such as EDTA and thiol-based compounds (2). The genes responsible for the production of MBLs are typically part of an integron structure and are carried on transferable plasmids but can also be part of the chromosome. Therefore, because of the integron-associated gene cassettes, *P. aeruginosa* isolates producing MBL are often resistant to different groups of antimicrobial agents which can be transferred to various types of bacteria (3).

Carbapenems (Imipenem and Meropenem) have a potent antipseudomonal activity and are often used as a last resort for the treatment of infections due to multi resistant *Pseudomonas* isolates. The introduction of carbapenems into clinical practice marked a great advance for the treatment of serious bacterial infections caused by beta-lactam resistant bacteria (4). *Pseudomonas aeruginosa* can develop resistance to carbapenems through diminished permeability, stable depression of chromosomal AmpC β-lactamases, or over-expression of the up-regulating efflux system. During the last decade, carbapenem resistance among hospital-acquired pseudomonas has been sporadically

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attributed to the production of the MBLs (5).

The most common and widespread acquired MBLs are those of the IMP and VIM types; both exhibit a worldwide distribution and for which several allelic variants are known. Acquired drug resistance is frequent in nosocomial isolates of *P. aeruginosa* and often involves more than one antibiotic class (6,7).

Infection with the MBL-producing organisms is associated with higher rates of mortality, morbidity, and health care costs. The international epidemiology of MBL-producing *P. aeruginosa* is still unknown in most countries (8), which is at least partly due to the lack of proper screening recommendations. For some countries, such Korea and Brazil, the proportion of MBL-producers among imipenem-resistant *P. aeruginosa* has been estimated to 11.4 and 20%, respectively (8). Nosocomial infection involving multiresistant *Pseudomonas aeruginosa* is a growing problem worldwide.

Rapid detection of MBLs is crucial for patient management and appropriate infection control procedures. The MBL enzyme detection in carbapenem-resistant *P. aeruginosa* was done using MBL screening tests: the imipenem/EDTA combined disc test (MBL-CD) which is based on the ability of EDTA to sequester zinc ions and to inactivate the metalloenzymes. Some studies of regional epidemiology have tried to address the issue of clonal dissemination, and although certain diversity can be observed in many areas, there is still evidence supporting that clonal expansion is an important mode of spread (9).

Materials and Methods

Bacterial isolates

Seventy-five isolates of *P. aeruginosa* were recovered from different clinical samples in Baghdad/Iraq during the period from April to August 2011. Isolates (75 isolates) were distributed as follows: burn (22), ear (14), sputum (13), wound (7), urine (5), blood (5), nasal swab (4), eye (3), a biopsy (2). Clinical samples were collected from teaching laboratories of the medical city, Baghdad, Al-Yarmouk Hospital, Al-Waseti Hospital, Al-Jadriya hospital, in addition to some private laboratories. Bacteria were cultured on MacConkey and Nutrient agar in aerobic condition at 42 °C for 24-48 h, and then identified by conventional biochemical tests and by using of VITEK 2 Automated system using (GN) cards.

Antibiotic susceptibility testing

All *P. aeruginosa* isolates were cultures on MacConkey agar supplemented with a Carbapenem (Meropenem) 4 mg/L (selective media) to check the susceptibility of *P. aeruginosa* isolates. VITEK 2 system using (AST- GN30) was used, and the MIC values for these isolates were obtained.

Identification of MBL- producing isolates

Imipenem-EDTA combined disc test (CDST) was used for identification of MBL-producing isolates according to Lee et al (10). The test organisms were inoculated on Mueller Hinton agar as recommended by the CLSI guidelines (11). A 0.5 M EDTA solution was prepared by dissolving 18.61 g. of EDTA in 100 ml of distilled water and adjusting its pH 8.0 by using NaOH. The Mixture was sterilized by autoclaving. Two imipenem (10 µg) discs were placed on the surface of an agar plate at a distance of 30 mm, and 4 µl EDTA solutions was added to one of them to obtain the desired concentration of 750 µg. The inhibition zones of imipenem and imipenem-EDTA discs were compared after 16 to 18 h of incubation in air at 37 ºC. In the combined disc test, if the increase in inhibition zone with the imipenem-EDTA disc was >7 mm than the zone of inhibition of imipenem alone, it was considered MBL positive (10).

Molecular detection of MBL genes

Isolation of genomic DNA from gram negative bacteria

This method was achieved according to the genomic DNA purification Kit supplemented by the manufacturing company (Intron, Korea).

Plasmid DNA extraction

DNA preparation from bacterial cells was performed by salting out method according to Sambrook and Russell (12) with some modifications as follows:

The bacterial cell of 50 ml culture was precipitated by centrifugation (1000 rpm for 10 minutes). Rewashed 3 times in TE buffer, and then the pellet was resuspended in 5 ml TE buffer. A volume of 600 µl of 25% SDS was added, mixed by inversion to the cell suspension and incubated for 5 minutes at 55º C. About 2 ml of 5M NaCl solution was added to the lysate, mixed thoroughly by inversion and let to be cooled to 37º C. Then 5 ml of (phenol: chloroform: iso amyl alcohol) (25: 24: 1 v/v) was added to the lysate and mixed by inversion for 30 minutes at 25º C and the spun by centrifuge 4500 rpm for 10 minutes. The aqueous phase was transferred to a fresh tube, which contains the nucleic acid then isopropanol (0.6 volume) was added to

the extract and mixed by inversion, after 3 minutes DNA spooled onto a sealed pasture pipette. The DNA rinsed in 5 ml of 70% ethanol, air dried, and dissolved in 300 µl TE buffer, and then DNA extract was kept at -20 oC until use.

Preparation of primers suspension

The DNA primers were resuspended by dissolving the lyophilized primers after spinning down with TE buffer depending on manufacturer instruction as stock suspension. Working primer tube was prepared by diluted with TE buffer. The final picomoles depended on the procedure of each primer.

Detection of MBLs genes by PCR

The MBLs and ESBLs genes were determined for the carbapenem resistant isolates by using targeting three genes, blaIMP, blaVIM, blaSPM-1. The PCR amplification mixture has been prepared according to the manufacturer's instructions (Intron, Korea).

Primers

The primers and PCR conditions used to amplify the genes encoding the MBLs enzymes are listed in Table-1.

Table 1. Primers sequences for detection of MBLs genes

Genes	Primer sequence (5'-3')	Product Size bp	Reference
*bla*IMPF	GAAGGCGTTTATGTTCATAC	587	(13)
*bla*IMPR	GTATGTTTCAAGAGTGATGC		
*bla*VIMF	GTTTGGTCGCATATCGCAAC	382	(13)
*bla*VIMR	AATGCGCAGCACCAGGATAG		
*bla*SPM-1F	CCTACAATCTAACGGCGACC	786	(13)
*bla*SPM-1R	TCGCCGTGTCCAGGTATAAC		

The reaction mixture

Amplification of DNA was carried out in a final volume of 20 µl. The contents of the reaction mixture were as follows: 2X PCR I-maxII master mix 4µl, Upstream primer1µl, Downstream primer1µl, DNA template 5µl, and Nuclease-free water 9µl. PCR product was detected using agarose gel electrophoresis.

Results

Out of the 75 *P. aeruginosa* isolates studied, only 16 (21.3%) isolates were able to grow on MacConkey agar supplemented with 2 mg/L carbapenem (meropenem). The antibiotic susceptibility test was performed for these isolates and 6 (8%) were carbapenems resistant, and 4 (5.33%) were MBL producers by phenotypic test (Table 2). In the present study, the MIC of 10 antibiotics listed in Table 3 was done using VITEK 2-Compact using AST-GN30 for testing the antibiotic susceptibility of these isolates. The MIC was done for 16 *P. aeruginosa* isolates that grow on MMAC, and the MIC values were interpreted according to the CLSI breakpoints.

Table 2. Antibiotic susceptibility of carbapenem-resistant *Pseudomonas aeruginosa* isolates

Isolate	Specimen	MBL CDST	MIC (µg/ml)of selected antibiotics determined by VITEK 2 system									
			IPM	SAM	FEP	FOX	CAZ	CRO	CIP	GM	MEM	TZP
Psa 3W	Wound	--	≥16 (R)	≥32 (R)	≥32 (R)	≥64 (R)	32 (R)	≥64 (R)	≥4 (R)	8 (I)	4(S)	≥128 (R)
Psa 8B	Burn	+	≥16 (R)	≥32 (R)	16 (I)	≥64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥16 (R)	≥16 (R)	≥128 (R)
Psa 9B	Burn	+	≥16 (R)	≥32 (R)	≥32 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥16 (R)	≥16 (R)	≥128 (R)
Psa 4E	Ear	--	≥16 (R)	≥32 (R)	16 (I)	≥64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥16 (R)	4 (S)	≥128 (R)
Psa 22B	Burn	+	≥16 (R)	≥32 (R)	≥32 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥16 (R)	≥16 (R)	≥128 (R)
Psa 12E	Ear	+	≥16 (R)	≥32 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥4 (R)	≥16 (R)	≥16 (R)	≥128 (R)

Abbreviations. IPM, imipenem; SAM, ampicillin/sulbactam; FEP, cefepime; FOX, cefoxitin; CAZ, ceftazidime; CRO, ceftriaxone; CIP, ciprofloxacin; GM, gentamicin; MEM, meropenem; TZP, piperacillin/tazobactam

The Carbapenem-resistant *P. aeruginosa* (CRPA) in this study (no. 6) differed in the level of resistance to different antibiotics including the carbapenems (Table 2). Four CRPA isolates showed MIC ≥16 µg/ml for both imipenem and Meropenem respectively while the remaining 2 CRPA were Meropenem susceptible MIC 4 µg/ml. The resistance profile of CRPA against the 4th generation of cephalosporins (cefepime) was different, 4 CRPA isolates were resistant all of these resistant isolates MIC 32-≥ 64 µg/ml while the other 2 isolates were intermediate to cefepime MIC 16 µg/ml. Only 10 (62.5 %) isolates out of 16 were resistant to cefepime. All the *P. aeruginosa* including the CRPA isolates were resistant to cefoxitin, ceftazidime, and ceftriaxone MIC 32-≥ 64 µg/ml.

The study showed that all CRPA isolates were resistant to ciprofloxacin MIC ≥4 µg/ml. Only one *P. aeruginosa* isolate (Psa-3W) showed intermediate resistance to aminoglycosides gentamicin MIC 8 µg/ml while the remaining CRPA *P. aeruginosa* isolates were resistant to gentamicin (Table 2). The detection of MBLs production among the CRPA isolates was done using combined-disc synergy test (CDST). Only 4 (5.33%) out of 75 *P. aeruginosa* isolates showed a positive result. As an inhibition zone with the imipenem-EDTA disc was >7 mm than the zone of inhibition of imipenem alone. These isolates were Psa8B, Psa 9B, Psa 22B, Psa12E, while the remaining Psa 4E, Psa 3W were negative as shown in Figure 1.

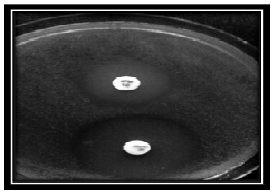

Figure 1. Combined disk synergy test (CDST) positive for MBL production in CRPA isolates, upper disk, is imipenem, and the lower disk is imipenem+EDTA.

Most of the carbapenems resistant *P. aeruginosa* isolates are taken from burned patients (Table 2). All the isolates are resistant to Imipenem MIC ≥16 while only 4 isolates are resistant to Meropenem MIC ≥16, and these isolates were found to be multi-drug resistant (MDR) to the 10 antibiotics (Table 2).

All *P. aeruginosa* isolates that were resistant to carbapenems (n=6) were further investigated for the presence of chromosomal or plasmid-mediated MBLs using 3 families of genes. The presence of MBL genes that include blaIMP, blaVIM, and blaSPM-1 was detected by conventional PCR technique.

PCR analysis for MBL genes was accomplished for the 6 CRPA isolates. The distribution of chromosomal and plasmid-mediated MBL genes within study isolates are shown in Figures 2 and 3. The CRPA isolates showing the variable presence of MBL genes. The genomic blaIMP was detected in 3 (50%) isolates; these are Psa 12E, Psa 9B, and Psa 22B, respectively. These results were achieved using specific blaIMP gene primers (Figure 2).

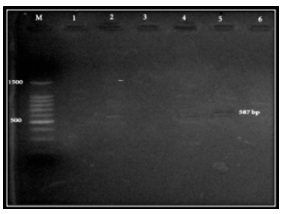

Figure 2. Ethidium bromide stained agarose gel showing PCR amplification products with *bla*IMP gene (587 bp) primers for *P. aeruginosa* extracted DNA.

M: 100 bp standard size reference marker, Lane 1: *P. aeruginosa* 4E shows negative result with *bla*IMP gene Lane 2: *P. aeruginosa* 12E shows positive result with *bla*IMP gene, Lane 3: *P. aeruginosa* 8B shows negative result with *bla*IMP gene, Lane 4: *P. aeruginosa* 9B shows positive result with *bla*IMP gene, Lane 5: *P. aeruginosa* 22B shows positive result with *bla*IMP gene, Lane 6: *P. aeruginosa* 3W shows negative result with *bla*IMP gene.

This study pointed out that no blaVIM gene was detected in any of CRPA isolates (Table 3). The present study indicated that only one CRPA isolate (Psa 9B) showed positive PCR amplification for the blaSPM-1 gene (16.6%) and this isolate Psa 9B also carried the blaIMP on their chromosomal DNA (Figure 3).

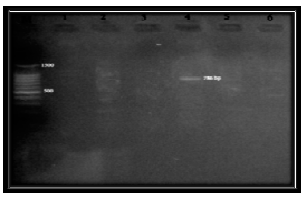

Figure 3. Ethidium bromide stained agarose gel showing PCR amplification products with *bla*SPM-1 gene (786 bp) primers for *P. aeruginosa* extracted DNA.

M: 100 bp standard size reference marker, Lane 1: *P. aeruginosa* 4E shows negative result with *bla*SPM1 gene Lane 2: *P. aeruginosa* 12E shows negative result with *bla*SPM-1 gene, Lane 3: *P. aeruginosa* 8B shows negative result with *bla*SPM-1 gene, Lane 4: *P. aeruginosa* 9B shows positive result with *bla*SPM-1 gene Lane 5:*P. aeruginosa* 22B shows the negative result with the *bla*SPM-1 gene, Lane 6: *P. aeruginosa* 3W shows the negative result with the *bla*SPM-1 gene.

Results also revealed that only 4 (66.6%) of CRPA isolates were identified as MBLs producer by CDST while the remaining two isolates identified as MBLs negative (33.3%). The overall picture of MBLs detection by phenotypic and molecular methods is summarized in Table 3.

Table 3. MBLs producing *P. aeruginosa* detected by phenotypic and molecular methods

Type of sample	No. of carbapenem-resistant	CDST*	*bla*IMP	*bla*VIM	*bla*SPM1
Burn	3	3 (100%)	2 (66.6%)	0 (0%)	1(50%)
Ear	2	1 (50%)	1 (50%)	0 (0%)	0 (0%)
Wound	1	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	6	4 (66.6%)	3 (50%)	0 (0%)	1 (16.6%)

Combined disk synergy test

Discussion

Pseudomonas is a common pathogen causing nosocomial infection. In the present study, MBL positive isolates show a high level of resistance to all β-lactam antibiotics including β-lactamase inhibitor, aminoglycosides, and quinolones. Resistance to aminoglycosides was present in most CRPA isolates but is not a reliable criterion for MBL-screening (13).

According to various studies, MBL production ranged from 7- 65%. In the Present study, MBL productions were 5.3% in isolates of *P. aeruginosa* using CDST (confirmative test). MBL positive isolates lead to serious therapeutic failure because they carry multidrug-resistant genes and the only treatment option available is potentially toxic polymyxin B (14). Out of 75 isolates of *Pseudomonas*, 6 (8%) were MBL positive by screening tests, 4 (5.3%) were MBL positive by CDST, as the CDST is more sensitive for detection of MBL producing isolates. It is necessary to detect MBL producing *Pseudomonas* isolates in routine clinical laboratory techniques by using MBL inhibitor (EDTA). Currently, no standardized method for MBL detection has been proposed and despite PCR being highly accurate and reliable, its accessibility is often limited to reference laboratories (15).

There is an increase in the resistance against the powerful carbapenems antibiotics. MBL positivity is increased dramatically among Imipenem and Meropenem resistant *P. aeruginosa* isolates. Long-term hospitalization, indwelling urinary catheters, and long-term antibiotic use (in particular of carbapenems) are the possible risk factors for colonization and/or infection such pathogens as MBLs positive *P. aeruginosa* (16).

In the current study, all CRPA isolates (No.= 6) were found to be resistant to at least 3, 4, and 5 antibiotic classes tested (100%) and considered as MDRs isolates (Table 2).

In the absence of novel antibiotics for the treatment of infections caused by multidrug-resistant gram-negative bacteria in the near future, uncontrolled spread of MBLs producers may lead to treatment failures with increased morbidity and mortality. Appropriate therapeutic protocols and a regular screening/monitoring system should be established to prevent the wider spread of this worrisome resistance determinant (16).

In 2004, most of the MDR *P. aeruginosa* did not carry MBL genes, and the resistance to imipenem might be attributed to the reduced expression of the OprD porin, combined with depression of the chromosomal ampC β-lactamase gene and/or overexpression of the efflux pump systems. However, in 2006 about 80% of the MDR *P. aeruginosa* were carriers of MBL genes that conferred the extrinsic drug resistance to imipenem (17).

The low susceptibility of the CRPA isolates against many antibiotics in this study may be due to extensive using of these antibiotics such as ciprofloxacin and gentamicin in clinical practice in Iraq. Excessive use of broad-spectrum antibiotics in hospitals has lead to the emergence of highly resistant strains of *P. aeruginosa*. To reduce the selection pressure for resistance, it is an important issue to determine the antibiotic susceptibility pattern of bacteria, so that hospital patients can be treated with more narrow-spectrum and target-specific antibiotics (18).

The results achieved by using PCR revealed that only 3 (50%) of CRPA isolates have MBLs genes. The MBLs gene was not detected in the remaining isolate that identified as MBLs producer by the phenotypic method. As the PCR is the gold standard technique. So, the phenotypic result may be a false positive result, or the isolates had MBLs variants or other carbapenemase genes not detected by the primers used in this study.

The blaVIM determinants have rapidly established a condition of high-level endemicity in Europe. Even the large outbreak reported in Greece was caused by a single clone and was apparently confined to the hospital wards (19). Gene encoding SPM-1 Metallo-β-lactamase was commonly found on the plasmid of several members of *P. aeruginosa*. However, this is the first attempt to characterize theblaSPM-1 gene within the plasmid of carbapenem-resistant *P. aeruginosa* isolates in Iraqi hospitals, whereas it was widely disseminated among Brazilian hospitals (20).

There is no information about the coexistence of two MBL genes in a single CRPA isolate; the explanation for such case is that Psa 9B isolate might take the chromosomal blaIMP gene from other Gram-negative bacteria by means of vertical gene transfer (VGT). Results also revealed that 3 (50%) of the 6 CRPA isolates obtained from different clinical samples carried blaIMP genes, followed by blaSPM-1 gene which obtained in 1 (16.6%) isolate. The blaVIM gene was negative in all CRPA isolates examined in this study. The gene encoding SPM-1, like both IMP and VIM, has been shown to be associated with a mobile genetic element and thus, may have originated in another bacterium (21).

Resistance to the carbapenems in the three (50%) MBLs negative *P. aeruginosa* in this study may be due to the impermeability, which arises via the loss of the OprD porin, the up-regulation of an active efflux pump system present in the cytoplasmic membrane of the bacteria. Metallo-β-lactamase encoded by genes carried on mobile genetic elements pose a greater clinical threat than chromosomally encoded enzymes, as these genes have the ability to transfer from one bacterium to another (21).

The prevalence of multi-drug resistant *P. aeruginosa* isolates especially carbapenem-resistant bacteria was higher than that which has been found in a survey of later years. The present study concluded that MBLs producing *P. aeruginosa* isolates were recognized in both phenotypic and molecular methods in local isolates recovered from health centers in Baghdad. The antibiotic resistance was increased against all third generation cephalosporins. Although carbapenem was the drug of choice for ESBL producing *P. aeruginosa* isolates, the emerging of MBLs producing bacteria poses a threat to an antibiotic treatment program in Baghdad hospitals. The blaIMP was the predominant among the MBLs genes of *P. aeruginosa* in this study. The blaIMP genes are encoded by chromosomal DNA while the blaSPM-1 encoded by plasmid and this resistance mechanism can cause nosocomial outbreaks.

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