ORIGINAL ARTICLE

# Prevalence of metallo-β-lactamase–producing *Pseudomonas aeruginosa* isolated from diabetic foot infections in Iraq

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## Abstract

Metallo-β-lactamase (MBL)-producing *Pseudomonas aeruginosa* is a major cause of nosocomial infections. However, there is little information in Iraq regarding its prevalence in patients with diabetic foot ulcer. Carbapenems are efficient antibiotics against extended-spectrum β-lactamase–producing *P. aeruginosa*. However, there are many potential health risks associated with carbapenem-resistant *P. aeruginosa*. We aimed to determine MBL-producing *P. aeruginosa* isolated from diabetic foot ulcer infections. A total of 97 *P. aeruginosa* isolates were isolated from pus and deep tissue swabs of 282 patients admitted to Al-Sader hospital, Najaf City, Iraq, with diabetic foot infections from October 2017 to January 2018. All *P. aeruginosa* isolates were tested by the Kirby-Bauer disc diffusion method for evaluating 13 antibiotics. Phenotypic carbapenem resistance was confirmed by the combined disc test, double-disc synergy test, modified Hodge test and CHROMagar KPC agar. All phenotypic MBL-producing *P. aeruginosa* isolates were screened for $bla_{IMP}$, $bla_{NDM}$, $bla_{SIM}$, $bla_{SPM}$ and $bla_{VIM}$ genes by multiplex PCR. Of the 97 *P. aeruginosa* isolates, combined disc test and modified Hodge test revealed 12 isolates (12.4%) to be MBL producers, and ten (10.3%) displayed MBL production as accessed by CHROMagar KPC agar test. Nine isolates (9.3%) were carbapenemase producers by the imipenem and ceftizoxime double-disc synergy test. Of 12 phenotypic MBL-producing *P. aeruginosa*, PCR amplification confirmed 4 (33.3%) and 3 (25%) isolates harbouring $bla_{VIM}$ and $bla_{IMP}$ gene respectively, but none carried the $bla_{NDM}$, $bla_{SIM}$ or $bla_{SPM}$ genes. The steady and rapid increase of MBL production is worrisome and needs to be controlled through extensive studies and more judicious selection of antibiotics, especially carbapenems.


**Keywords:** Diabetic foot infections, metallo-β-lactamase, *pseudomonas aeruginosa*
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## Introduction

Diabetes mellitus is a disorder that affects many people as a complex and serious disease [1,2]. More than 439 million people are diagnosed with diabetes mellitus, which causes a high rate of mortality and morbidity around the world [1]. Diabetic foot ulcer (DFU) is an important cause of complications of diabetes mellitus, which leads to the development of wet gangrene, causing inevitable limb amputation [2]. Determination of causative agents is crucial to selecting appropriate and accurate therapy. Various pathogens have been isolated, depending on local studies and geographic variations. Although in North America and Europe Gram-positive species like *Staphylococcus aureus* are predominant, in Asia Gram-negative species like *Pseudomonas aeruginosa* are predominant [3,4]. The complications of multidrug-resistant (MDR) *P. aeruginosa* have caused a major health concern among patients with DFU. In the most recent decade, high rates of metallo-β-lactamase (MBL)-producing *P. aeruginosa* have been observed in many hospitalized patients with DFU, leading to lower-extremity amputation [3]. Major mechanisms of carbapenem resistance among *P. aeruginosa* include the loss of porin OprD and production of carbapenemases. Carbapenem nonsusceptibility, particularly facilitated through plasmids and extrachromosomal



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elements, has limited therapeutic options against serious Gram-negative infections [4,5]. Considering the vulnerability of patients with DFU, early diagnosis of DFU and appropriate selection of antimicrobial therapy are essential for controlling diabetic foot infections and preventing complications.

We evaluated the MBL-producing MDR *P. aeruginosa* in infected DFUs and their sensitivity profiles in Al-Najaf City, Iraq.

## Patients and methods

### Patients

The study population was defined as 282 patients with DFU infections admitted to Al-Sader Hospital, Al-Najaf City, Iraq, from October 2017 to January 2018. Information was gathered regarding patient demographic and clinical features such as gender, age, type of diabetes, wound size, random blood sugar level, nature of ulcer, location of the lesion and amputation.

### Characterization of bacterial isolates

Wound exudates and pus were obtained using deep swab techniques of the wound area. The specimens were inoculated on brain–heart infusion agar, MacConkey agar and blood agar (HiMedia, Mumbai, India) plates for the isolation bacteria. The individual colonies were isolated, and the identification was performed according to our standard medical microbiology laboratory's analysis of DFU [2].

### Antibiotic susceptibility patterns

Susceptibility of 97 isolates to antipseudomonal antibiotics was determined by the disc diffusion method. *In vitro* susceptibility test was performed for 13 antipseudomonal agents: piperacillin (PI, 75 μg), piperacillin/tazobactam (PIT, 100 μg), ceftazidime (CA, 30 μg), ceftizoxime (CZX, 30 μg), aztreonam (AT, 30 μg), imipenem (IPM, 10 μg), polymyxin B (PB, 300 U), gentamicin (GEN, 10 μg), colistin (Col, 10 μg), tobramycin (TOB, 10 μg), amikacin (AK, 30), ciprofloxacin (CIP, 5 μg) and levofloxacin (LE, 5 μg) (HiMedia). Analysis of each isolate was conducted by modifying the Kirby-Bauer method. The zone of inhibition was measured according to recommendations by the Clinical and Laboratory Standards Institute (CLSI) [6].

### Minimum inhibitory concentrations

The imipenem and meropenem MIC was determined using the agar dilution method according to CLSI 2017.

### Phenotypic detection of MBL

*Modified Hodge test.* The turbidity of *Escherichia coli* ATCC 25922 inoculum was standardized to match 0.5 McFarland and streaked on to a Müller-Hinton agar (MHA) plate as a lawn. The imipenem disc was placed exactly in the middle of the lawn. Each test isolate was seeded carefully in a straight line from edge to edge between the imipenem disc and the lawn plate. Appearance of a cloverleaf shape was considered MBL positive [6].

*Carba-NP test.* The Carba-NP test was implemented according to CLSI 2017. Briefly, $DDH_2O$, phenol red and $MgSO_4$ were mixed, and imipenem was added. Next, after being formed in to aliquots, 100 μL of overnight colony culture of isolate was inoculated in the tube and incubated for 2 to 4 hours. Carbapenemase-producing isolates would alter the colour to yellow.

*Combined disc test.* The inoculum of test isolate was diluted by adjusting the 0.5 McFarland turbidity and was inoculated on a MHA plate. The imipenem (10 μg) disc was combined with 8 μL EDTA, and the ceftizoxime (30 μg) disc was also combined with 8 μL EDTA. The distances between the substrates and the inhibitor discs from center to center were tested as follows: 1, 1.5, 2, 2.5 and 3 cm. The appearance of an enhanced zone $\geq$8 mm between the substrate and inhibitor discs compared to the substrate discs alone was considered to be a positive result for MBL production [7,8].

*Double-disc synergy test.* Imipenem (10 μg) and EDTA (750 μg) discs were carefully placed on inoculated MHA with the test isolate. The space between the center of the imipenem and EDTA discs was 20 mm. Enhancement of the inhibition zone in the distance between both discs compared to the inhibition zone on the far side of imipenem disc was reported as an MBL-positive result [9].

*Streaking on CHROMagar KPC agar.* All isolates were streaked on CHROMagar KPC agar and incubated at 37°C overnight, according to the manufacturer's instructions. MBL-producing *P. aeruginosa* colonies appeared translucent cream to blue.

### Molecular methods

DNA was extracted according to the instruction of the Genomic DNA Mini Kit manufacturer (Geneaid, New Taipei City, Taiwan). PCR detection of the $bla_{IMP}$, $bla_{NDM}$, $bla_{SIM}$, $bla_{SPM}$ and $bla_{VIM}$ genes was conducted using primers described previously [10]. PCR was performed with 12.5 μL of master mix, 5 μL DNA template and 0.5 μL of each primer (Kapa, Cape Town, South Africa) containing 1 U of Taq DNA polymerase in a total final volume of 20 μL. A thermocycler instrument (A&B, Singapore) was used with the reaction conditions (Table 1).

## Results

Of 282 pus and exudate specimens screened, 97 (34.4%) *P. aeruginosa* were identified, of which 54 (55.7%) occurred in male and 43 (44.3%) in female subjects. The female-to-male ratio

**TABLE 1. Primers used in multiplex PCR for determining MBL-producing *Pseudomonas aeruginosa* isolates**

| Target gene | Direction | Primer sequence (5′→3′) | Amplicon size (bp) |
|---|---|---|---|
| $bla_{IMP}$ | F | GGAATAGAGTGGCTTAAYTCTC | 232 |
| | R | GGTTTAAYAAAACAACCACC | |
| $bla_{NDM}$ | F | GGTTTGGCGATCTGGTTTTC | 621 |
| | R | TGGGTRAARTARGTSACCAGA | |
| $bla_{SIM}$ | F | ACATTATCCGCTGGAACAGG | 570 |
| | R | TACAAGGGATTCGGCATCG | |
| $bla_{SPM}$ | F | CGAATGCGCAGCACCAG | 271 |
| | R | AAAATCTGGGTACGCAAACG | |
| $bla_{VIM}$ | F | GATGGTGTTTGGTCGCATA | 390 |
| | R | CGAATGCGCAGCACCAG | |

Reaction conditions and steps were as follows: initial denaturation, 94°C for 10 minutes; denaturation, 94°C for 30 seconds; annealing, 52°C for 40 seconds; extension, 72°C for 50 seconds; final extension, 72°C for 5 minutes; for 32 cycles.
F, forward; MBL, metallo-β-lactamase; R, reverse.

was 0.8. Age ranged from 30 to 70 years ( mean, 59 ± SD 3.3 years). The prevalence of age groups is shown in Fig. 1. In this study, 79 (81.44%) of 97 of *P. aeruginosa* were isolated from Wagner DFU wound grades II and III [2]. Seventy-six patients (78.4%) received antibiotics alone, whereas 21 (21.6%) underwent a surgical procedure with multiple antibiotics.

### Antimicrobial susceptibility test

The antibiotic susceptibility of isolates was shown in Table 2. All isolates were sensitive to colistin and polymyxin B. Isolates expressed varying degrees of resistance to aminoglycosides (18.6–28.9%) and fluoroquinolones (19.6–22.7%). Notably, 12 isolates (12.4%) were MDR, being resistant to four classes of antibiotics; all these isolates were MBL producers.

### Phenotypic detection of carbapenemase production

In this study, screening for MBL production using the Carba-NP test, combined disc test and modified Hodge test was performed, with 12 (12.4%) among which Carba-NP test had higher sensitivity. Ten isolates (10.3%) had positive result by CHROMagar KPC agar testing, whereas nine isolates (9.3%) were carbapenemase producers by the imipenem and cefotizoxime double-disc synergy test (Figs. 2 and 3).

**TABLE 2. Antimicrobial susceptibility results of *Pseudomonas aeruginosa* isolates**

| Antimicrobial class | Antimicrobial agent | *N* (%) *P. aeruginosa* isolates that are: R | I | S |
|---|---|---|---|---|
| Penicillins | Piperacillin | 45 (46.4) | 0 | 52 (53.6) |
| β-Lactam combination agents | Piperacillin/tazobactam | 41 (42.3) | 0 | 56 (57.7) |
| Cephems | Ceftazidime | 40 (41.2) | 0 | 57 (58.7) |
| | Ceftizoxime | 42 (43.3) | 0 | 55 (56.7) |
| Monobactams | Aztreonam | 39 (40.2) | 0 | 58 (50.8) |
| Carbapenems | Imipenem | 12 (12.4) | 0 | 85 (87.6) |
| Lipopeptides | Colistin | 0 | 0 | 97 (100) |
| | Polymyxin B | 0 | 0 | 97 (100) |
| Aminoglycosides | Gentamicin | 28 (28.9) | 4 (4.1) | 65 (67.0) |
| | Tobramycin | 24 (24.7) | 3 (3.1) | 70 (72.2) |
| | Amikacin | 18 (18.6) | 1 (1.0) | 78 (80.4) |
| Fluoroquinolones | Ciprofloxacin | 22 (22.7) | 2 (2.1) | 73 (75.3) |
| | Levofloxacin | 19 (19.6) | 2 (2.1) | 76 (78.4) |

I, intermediate; R, resistant; S, sensitive.

### Molecular detection of carbapenemases

Of 12 phenotypically MBL-producing *P. aeruginosa* isolates, four (33.3%) and three (25%) isolates harboured $bla_{VIM}$ and $bla_{IMP}$ genes respectively. Additionally, two isolates carried the $bla_{SPM}$ and $bla_{SIM}$ genes, and one carried the $bla_{NDM}$ gene. Multiple MBL gene carriage is shown in Table 3.

Regarding receipt of prior antibiotic therapy, six of 12 and three of 12 had a history of β-lactam and fluoroquinolone receipt respectively.

## Discussion

The inherent and extensive antibiotic resistance of *P. aeruginosa* has restricted therapeutic choices, necessitating proper considerations in tissue damage in DFU patients [5,11]. Carbapenem resistance has been widely studied around the world. However, local studies are scarce, and they fail to address the extent of the serious problem facing the health sector in our country. One of the local studies conducted by Al-Charrakh et al. [12] reported that 37.5% of MBL producers in different clinical samples were isolated from hospitals in Baghdad. Another study conducted by Yassin et al. [13] reported that 12.7% of MBL-producing *P. aeruginosa* were isolated from wound samples at Duhok Hospital, Iraq, and a low prevalence 3.95% of MBL producers was reported by Anoar et al. [14] from patients with burn infections in Sulaimani City, Iraq. To our knowledge, there is no local study about the MBL-producing *P. aeruginosa* rate from diabetic foot infections in Iraq or in Al-Najaf City.

The prevalence of MBL in the current study was 12 (12.4%) among the 97 *P. aeruginosa* isolates. It was nearly similar to the rates in the other studies, such as the prevalence of MBL producers of 10% in India, 12% in Canada, 12.7% in the United Arab Emirates, 13.4% in Russia and 14% in Spain [13,15]. Some studies have recorded various percentages of MBL-producing *P. aeruginosa*, such as 38.3% in São Luis of Brazil, 47.3% in Taiwan, 62% in Greece [16] and 53.2% in Iran [17]. Large outbreaks caused by carbapenem-resistant strains have been reported in Greece, Korea, Kenya, Canada and Italy [18,19]. The frequency of carbapenem-resistant *P. aeruginosa* was increased from 1% to 28% between 2002 and 2006, particularly in Europe [20,21].

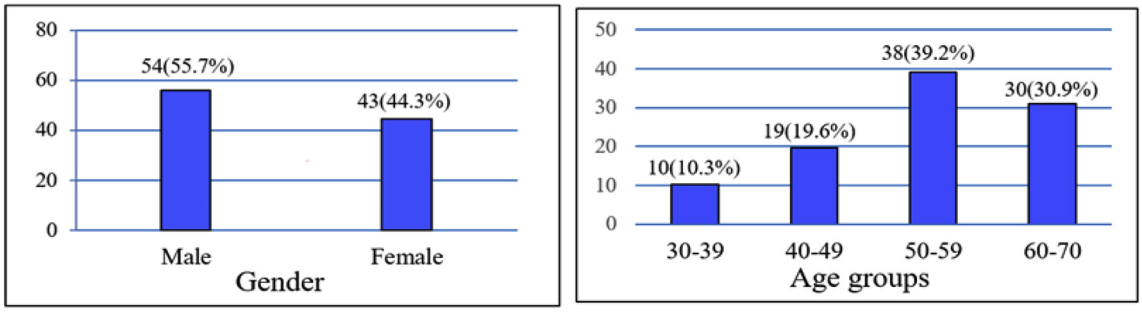


**FIG. 1.** Histograms showing distribution of *Pseudomonas aeruginosa* by (left) sex and (right) age.

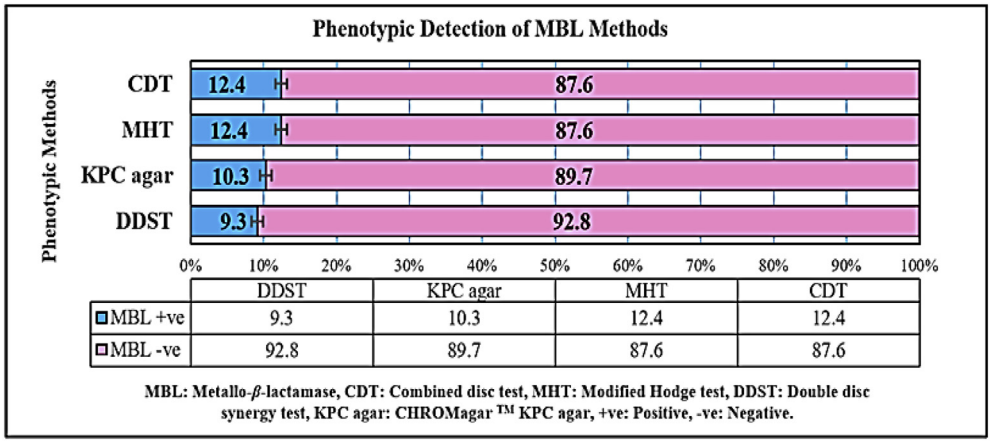


**FIG. 2.** Phenotypic detection of carbapenem resistance among *Pseudomonas aeruginosa* isolated from patients with diabetic foot infections.

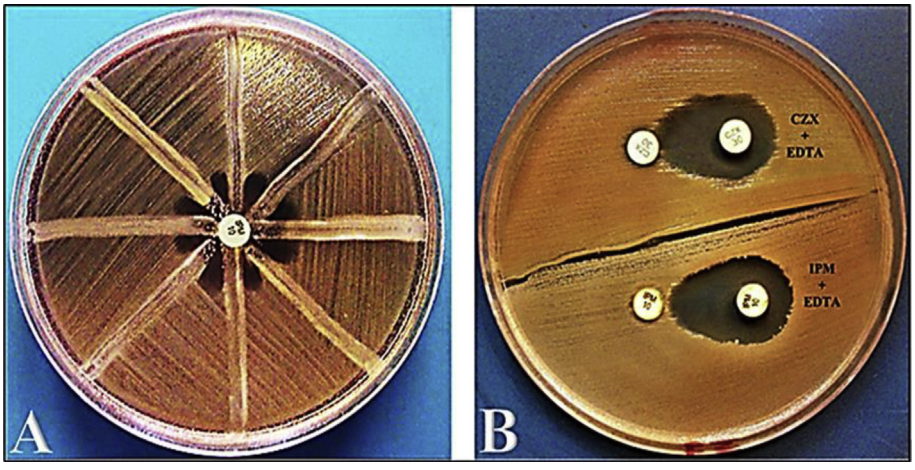


**FIG. 3.** Phenotypic detection of metallo-β-lactamase by various tests. (A) Modified Hodge test had positive result, giving rise to a cloverleaf pattern around imipenem (IPM, 10 μg) disc by isolates 7, 13, 22, 23, 43, 57 and 83. (B) Combined disc test's imipenem disc produced large synergistic inhibition zone towards imipenem (10 μg/8 μL EDTA) disc by isolate no. 22 (top half of plate); ceftizoxime (CZX, 30 μg) disc produced large synergistic inhibition zone towards ceftizoxime (30 μg/8 μL EDTA) disc by isolate no. 7 (bottom half of plate).

Although the results of our phenotypic methods were not entirely the same, they were convergent and can be applied in a complementary manner to molecular methods [22,23]. We had a limitation regarding lack of MIC of imipenem associated with the existence of genes.

All MBL-producing *P. aeruginosa* isolates were MDR and were resistant to β-lactams, aminoglycosides and fluoroquinolones.

The prevalence of the VIM and IMP genes in this study was 33.3% and 25% respectively, which was similar to the results of

**TABLE 3. Characteristics of 12 multidrug-resistant *Pseudomonas aeruginosa* isolates**

| Isolate no. | Sex (age in years) | $bla_{VIM}$ | $bla_{IMP}$ | $bla_{NDM}$ | $bla_{SIM}$ | $bla_{SPM}$ |
|---|---|---|---|---|---|---|
| 1 | F (51) | | | | | |
| 2 | F (49) | + | + | | | |
| 3 | M (36) | | | | + | |
| 4 | M (68) | | + | | | + |
| 5 | M (48) | | | | | |
| 6 | F (57) | | | | + | |
| 7 | M (62) | + | | | | |
| 8 | F (65) | + | | | | + |
| 9 | F (48) | | | | | |
| 10 | M (53) | | + | | | |
| 11 | M (49) | | | | | |
| 12 | M (67) | + | | + | | |

the other studies that have demonstrated that VIM and IMP are prevalent and common in Asian countries, Spain and other areas [24,25]. Similar results have been observed in some studies conducted in Iraq's neighbouring countries such as Iran. For example, in the study by Azimi et al. [26], the common gene among MBL producers was IMP, which occurred at 17.5%, followed by 15.6% for the VIM gene. In another study from Iran, Salimi and Eftekhar [27] found VIM-1 and IMP-1 genes to be more common than other MBL-encoding genes. Saffari et al. [28] reported that 18% and 5.5% of MBL-producing *P. aeruginosa* were positive for the IMP and VIM genes, respectively. Radan et al. [29] documented that 74.3% of the MBL-producing isolates carried the IMP gene. Azimi et al. [30] believed the increase and widespread of the IMP and VIM genes among clinical *P. aeruginosa* contributed to several unpleasant nosocomial and healthcare-associated infections, the result of the potential spread of virulence genes among bacterial species through effective mechanisms such as horizontal gene transfer.

Several studies have demonstrated that the VIM gene in *P. aeruginosa* is the predominant MBL in Iraq [31]. These local studies determined the rate of the VIM and IMP genes to be 85% and 57% in Erbil [32]. However, the VIM gene was highly (94.4%) reported in Wasit [33], whereas Al-Charrakh et al. [12] detected no VIM gene in their study.

We observed that among patients infected with MBL-producing *P. aeruginosa*, six of 12 and three of 12 had a history of receipt of β-lactams and fluoroquinolone respectively.

However, studies in Columbia, Italy and Japan have revealed that regardless of prior antibiotic receipt, *P. aeruginosa* isolates carry the VIM and IMP genes [16,34]. It is notable that isolates harbouring the VIM and IMP genes are also resistant to quinolones, aminoglycosides and sulfonamides [16,25,35]. This phenotype is due to the carriage of mobile genetic cassettes and other determinants of resistance inserted into integrons [25].

## Conclusion

The steady rapid increase of MBL production among nosocomial *P. aeruginosa* isolates is worrisome and needs to be controlled through urgent and extensive studies. All our isolates were susceptible to colistin and polymyxin B.

## Conflict of interest

None declared.

## Acknowledgements

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