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 blaIMP, blaNDM, blaSIM, blaSPM and blaVIM 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 blaVIM and blaIMP gene respectively, but none carried the blaNDM, blaSIM or blaSPM 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.

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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...
elements, has limited therapeutic options against serious Gram-
negative infections [4,5]. Considering the vulnerability of pa-
tients with DFU, early diagnosis of DFU and appropriate se-
lection 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
onto a Müller–Hinton agar (MHA) plate as a lawn. The imipen-
em 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, DDH2O, phenol red and MgSO4 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 ≥8 mm between the sub-
strate 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)
disks 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, ac-
cording 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 blaSPM, blaNDM, blaIMP, blaVIM,
and blavIM 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) |
|-------------|-----------|-------------------------|-------------------|
| blaSIM | F | GGAATAGAGTGCTAATTAATCCT | 232 |
| R | AAAATCTGGGTACGCAAACG |
| blaSHV | F | GGGTGGCGCTGTTGTTTTC | 621 |
| R | TGGGTAATGATGTGCAAGCAGA |
| blaIMP | F | ACATTACGGTGGAAACGG | 570 |
| R | TACAGGAGTCGCAATCG |
| blaVIM | F | CGAATGCGCACGACACG | 271 |
| R | AAAAACTTTGAGTCCAAACAGC |
| blaVBP | F | GATGGTGGTTGGTCGATA | 390 |
| R | CGAATGCGCACGACACG |

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 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].

### 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.

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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

![FIG. 1. Histograms showing distribution of *Pseudomonas aeruginosa* by (left) sex and (right) age.](image1)

![FIG. 2. Phenotypic detection of carbapenem resistance among *Pseudomonas aeruginosa* isolated from patients with diabetic foot infections.](image2)

![FIG. 3. Phenotypic detection of metallo-β-lactamase by various tests.](image3)
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, Salmi and Eftekhar [27] found VIM-I and IMP-I 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 VIM 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.

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