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bla-VIM Gene among Metalo-β-Lactamase Producing *Pseudomonas aeruginosa*

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Abstract

The aims of this study were to determine the antibiotic resistance patterns in *Pseudomonas aeruginosa* species isolated from three hospitals and to determine the prevalence of metalo-β-lactamase (MBL) *bla-VIM* encoding gene. A total of 134 clinical isolates of *P. aeruginosa* were collected from different pathological specimens over 12 months. Phenotypic characterization was performed using EDTA and MBL E-test strip. MBL producing *P. aeruginosa* isolates were further screened for the *bla-VIM* gene by polymerase chain reaction.

All isolates were resistant to the antibiotics tested including aminoglycosides, fluoroquinolones and carbapenems, but susceptible to colistin. Phenotypic screening for MBL production indicated that 31.3% were MBL positive. Imipenem-resistant isolates (MIC of > 8 μg/ml) (51/73; 71.2%) were MBL positive; the majority (34/42; 81%) were isolated from the burns unit. VIM-positive isolates (20.1%) had significantly ($P \leq 0.05$) higher proportions of resistance to certain antibiotics carbapenems, aminoglycosides, fluoroquinolones, cefepime and piperacillin-tazobactam compared to VIM-negative isolates. The proportion of MDR isolates was 53%, mainly obtained from patients in ICU patients with burn and significantly higher ($P = 0.003$) among VIM producers. It was observed that all isolates that were positive for MBL E-test and EDTA had the *bla-VIM* gene and were MDR isolates. These were found only among isolates from ICU patients with burns.

The high rate of antibiotic resistance among *P. aeruginosa* strains expressing *bla-VIM* gene is alarming and can be responsible for serious infections especially among burns patients; therefore, guidelines and appropriate infection control measures needed to be established to reduce these infections among patients.

Keywords: *P. aeruginosa; Antibiotics; Metalo-β-Lactamase; bla-VIM Gene; Libya*

Introduction

Pseudomonas aeruginosa is one of the causative agents of hospital-acquired infections, especially in patients with burns [1]; both intrinsic and acquired resistance to antibiotics complicates antipseudomonal chemotherapy. Carbapenems have been the drug of choice for the treatment of infections caused by penicillin or cephalosporin resistant Gram-negative bacilli [2]; however, carbapenem resistance has been observed frequently in *P. aeruginosa*. The broad-spectrum resistance of *P. aeruginosa* is reflect to a combination of factors: low outer membrane permeability; the presence of the inducible AmpC chromosomal β-lactamase; synergistic action of several multidrug efflux systems and the presence of transferable resistance determinants, in particular, carbapenem-hydrolyzing enzymes, mainly metallo β-lactamases (MBLs); and aminoglycoside-hydrolyzing enzymes [3].

The most common MBLs found in *P. aeruginosa* include VIM, IMP, GIM, FIM-1, and SPM. In particular, *bla-VIM*-2 has emerged as a dominant MBL variant in worldwide [4]. The VIM types have been identified in carbapenem-resistant isolates of *P. aeruginosa* from African countries (Libya, Tunisia, Algeria and Egypt) [5-8] and from countries in the Mediterranean basin (France, Lebanon and Spain) [9-11]. *P. aeruginosa* with VIM-2 has been imported from African countries (Ghana, Tunisia, and Egypt) into Europe [12-14].

Immediate detection of MBL-producing *P. aeruginosa* is important to prevent the spread of the organism within and between hospitals, and to treatment of immunocompromised and critically ill patients. Various methods have been suggested for screening of MBL-producing *P. aeruginosa* including phenotypic and molecular techniques. To date, few studies have been carried out in Libya to establish the associations between clinical origin of strains, resistance-encoding phenotypes, and phylogenetic groups among *P. aeruginosa* isolates [5,15]. The aims of this study were to determine the antibiotic resistance patterns in *P. aeruginosa* species isolated from three hospitals in Tripoli and to evaluate the prevalence of MBLs *bla-VIM* encoding gene using polymerase chain reaction (PCR).

Materials and Methods

P. aeruginosa isolates

A total of 134 non-duplicate, nonconsecutive clinical isolates of *P. aeruginosa* were collected over 12 months between April 2013 and April 2014 from three hospitals. Burn and Plastic Surgery Centre (BPSC) is a 120 bed hospital that provides medical care for burn patients with separate male, female and children's burn and plastic surgery units. Tripoli Central Hospital (TCH) is a 640-bed university affiliated tertiary hospital. Tripoli Medical Center (TMC) is a tertiary teaching hospital with a capacity of 1200 beds. The patients included 71 males and 63 females aged 1-83 years old (mean = 33.8 years). Organisms were isolated from different pathological specimens, primarily swabs from anatomical sites (n = 87), urine (n = 24), blood (n = 17) and unspecified (n = 6). The majority were obtained from ICUs (77/134; 57.7%). All specimens were taken as part of the clinical workup in this prospective laboratory-based surveillance study. In this investigation, specimens were collected under approved ethical standards and the study was reviewed and approved by the Faculty of Pharmacy, University of Tripoli and hospitals participating in this study.

Antibiotic susceptibility testing

All specimens were cultured on appropriated media by standard bacteriological procedures. Isolated organisms were identified to species level and tested for their susceptibility to a variety of antimicrobial agents (Table 1) by the BD Phoenix Automated Microbiology System (PAMS, MSBD Biosciences, Sparks Md, USA) according to the manufacturer's instructions. All isolates were screened and interpreted for ESBL phenotype according to the criteria of the CLSI [16]. Multidrug resistant organisms (MDR) were defined as showing resistance to three or more different classes of antibiotics including fluoroquinolones, aminoglycosides, carbapenems and cephalosporins. Reference strain of *E. coli* ATCC 25922, *E. coli* ATCC 35218, *Klebsiella pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853 were used as controls.

Phenotypic detection of carbapenemases

Isolates were screened for carbapenemase production using E-test (Liofilchem, Rosetodegli Abruzzi, Italy) as recommended by manufacturer and the ethylenediaminetetraacetic acid sodium mercaptoacetic acid (EDTA) disk synergy test, as previously described [17].

Molecular detection of *bla*-VIM gene

MBL producing *P. aeruginosa* isolates were screened for the *bla-VIM* gene using previously reported primers [18]. The plasmids were isolated using the QIAGEN Plasmid Mini Kit according to the manufacturer's instructions. The reaction mixture contained a total of 25 µl: 5 µl of 5X Red Load Taq Mix composed of Taq Polymerase, 0.05 u/µl dNTPs (200 µM) (dATP, dCTP, dGTP, dTTP) reaction buffer with KCl and MgCl2 (1.5 mM) red dye, gel loading buffer, stabilizers (Metabion, Martinsried- Germany); 0.5 µl of each primer 10 pmol/µl; and extracted plasmid DNA (2 - 50 ng). The thermal profile included one cycle of initial denaturation at 95oC for 2 minutes followed by 35 cycles at 95oC for 30 seconds, annealing at 52oC for 30 seconds, and extensions at 72oC for 45 seconds. The PCR reaction was carried out

with TC-412 thermocycler (Techne, Duxford, Cambridge). Five µl of the PCR amplification products were electrophoresed in agarose (2% m/v) containing 0.5 ug/mL ethidium bromide. The amplified PCR products were visualized under UV light and electronically documented with a gel documentation system (MultiDoc-It Digital Imaging System UVP, Cambridge, UK). A 50bp DNA ladder (Metabion, Martinsried-Germany) was used as a molecular size marker.

Statistical analysis

All statistical analysis was performed using the statistical package software SPSS 16 (SPSS Inc, Chicago, IL). Statistical analyses were performed using the chi-square test. The statistical significance was set at P = 0.05.

Results

A total of 134 non-duplicate isolates were collected and confirmed as *P. aeruginosa* by the Phoenix automated system. The results of antibiotic susceptibility testing revealed that the isolates were highly resistant to tested antibiotics: β-lactams (33.6% - 68.6%), aminoglycosides (43.3% - 61.2%), and fluoroquinolones (64.2% - 71.6%). All isolates were susceptible to colistin. Phenotypic screening for MBL production among *P. aeruginosa* isolates found that 42/134 (31.3%) were MBL positive based on MBL E-test and the activity of β-lactams was inhibited by EDTA synergistic test demonstrated that (52/134; 38.8%). Imipenem-resistant (all showed MIC of > 8 µg/ml) *P. aeruginosa* isolates (51/73; 71.2%) were MBL positive; the majority (34/42; 81%) of these strains were isolated from burn patients. The antipseudomonal antibiotics (cefepime, ceftazidime, imipenem, piperacillin-tazobactam) showed a different degree of resistance (68.7%, 63.4%, 55.5% and 33.6%, respectively). The highest proportions of isolates resistance to these antibiotics were detected in those from specimens from burns patients. The proportion of MDR organisms was (71/134; 53%) and mainly associated with ICU patients of the burns hospital. PCR was performed to determine the presence of the *bla-VIM* gene among all phenotypically MBL-positive isolates (Figure 1). The results of antimicrobial susceptibility tests for VIM-positive (27/134; 20.1%) strains and VIM negative (107/134; 79.9%) are summarized in table 1. VIM-positive isolates had significantly higher proportions resistant to antibiotics such as carbapenems, aminoglycosides, fluoroquinolones, cefepime and piperacillin-tazobactam compared to VIM-negative isolates (P ≤ 0.05) (Table 1). The proportion of MDR *P. aeruginosa* was significantly higher (P = 0.003) among VIM producers compared with non-producers. It was observed for that all isolates that positive for MBL by E-test and EDTA were found to have the *bla-VIM* gene and were MDR isolates. All of these isolates that harbored *bla-VIM* gene were detected from BPSC, the majority of these isolates 23/27 (85.2%) were recovered from the patient wounds in the burn ICU; four isolates expressing *bla-VIM* gene were obtained from blood of burn patients.

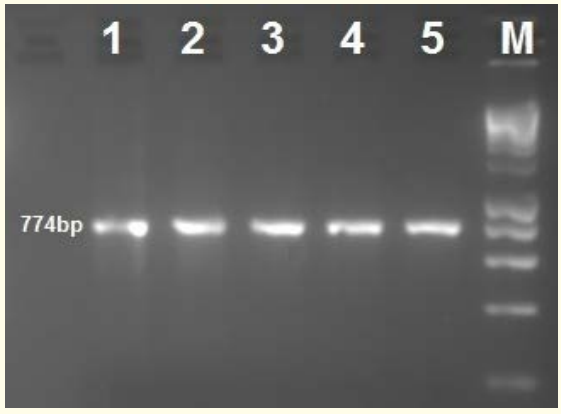

Figure 1: *Amplification of bla-VIM gene in MBL producing P. aeruginosa isolates by single PCR. Lanes 1 to 5 bla-VIM-producing isolates; M: Smart Ladder. The amplified amplicon size is 774bp*

Antibiotic	VIM-positive *n* = 27 (%)	VIM-negative *n* = 107 (%)	P value
Cefoxitin	27 (100)	107 (100)	0.5
Cefuroxime	27 (100)	107 (100)	0.5
Cephalothin	27 (100)	107 (100)	0.5
Ceftazidime	27 (100)	58 (54.2%)	0.008
Meropenem	26 (96.3)	42 (39.3)	0.0004
Imipenem	27 (100)	46 (43)	0.003
Ertapenem	27 (100)	107 (100)	0.5
Gentamicin	25 (92.6)	57 (53.3)	0.002
Amikacin	22 (81.5)	36 (33.6)	0.0001
Levofloxacin	26 (96.3)	60 (56.1)	0.004
Ciprofloxacin	26 (96.3)	67 (62.6)	0.008
Nitrofurantoin	27 (100)	107 (100)	0.5
Trimethoprim-Sul	27 (100)	107 (100)	0.5
Ampicillin	27 (100)	107 (100)	0.5
Aztreonam	20 (74.1)	58 (54.2)	0.07
Cefepime	27 (100)	65 (60.7)	0.01
Colistin	0 (0)	0 (0)	0.5
Piperacillin-Tazobactam	15 (55.5)	30 (28)	0.009
Amoxicillin-Clavulanate	27 (100)	107 (100)	0.5
MDR	27 (100)	44 (41.1)	0.003

Table 1: *Antimicrobial resistance patterns of P. aeruginosa clinical isolates.*

Discussion

MBLs have recently been identified in clinical isolates with increasing frequency across the world and *P. aeruginosa* strains that produce these enzymes have been responsible for prolonged treatment and acute infections [19]. Detection of MBLs producing strains is needed for optimal treatment of patients particularly in burned and hospitalized patients and for control of the spread of resistance [20,21]. In this study, we investigated the prevalence of the *bla-VIM* gene among *P. aeruginosa* isolates recovered from three major Tripoli hospitals.

The rate of imipenem-resistant *P. aeruginosa* isolated from different healthcare setting in Libya has increased consistently from 8.3% in 2012 to 36% in 2015 [22-25]. We observed high rates of resistance to commonly used carbapenem agents in Libya (meropenem 50.7% and imipenem 54.5%); increase in of carbapenem-resistance reflects a threat to treatment options in our hospitals especially for burn patients. In the Middle East the occurrence of imipenem-resistant *P. aeruginosa* is increasing [26]. Similar findings were reported by Sheikh and co-workers, who detected high rate of resistance to imipenem (58.7%) in isolates recovered from two general Iranian hospitals and one burn hospital [27].

Piperacillin-tazobactam showed enhanced activity against tested isolates (66.4%) compared with other antipseudomonal agents including cefepime, ceftazidime and imipenem (31.3% - 45.5%). The prevalence of the various patterns of drug resistance, especially MBL encoding genes, can be different among *P. aeruginosa* isolates from one region to another or even between different hospitals in the same area [28]. Overall, the rate of MDR isolates was 53% and mainly associated with ICU burn patients. MDR *P. aeruginosa* isolates have been detected in hospitals worldwide and associated with increased mortality and costs due to prolonged hospitalization, need for surgery, and prolonged treatment with antibiotics [29].

In the present study, phenotypic screening for MBL production among *P. aeruginosa* isolates based on MBL E-test and the EDTA synergistic test was 31.3% and 38.8% respectively. Meradji., *et a*l. examined carbapenem resistant *P. aeruginosa* isolated from different clinical specimens from three hospitals in Annaba City, Algeria. They did not detect MBLs in any isolates using phenotypic and PCR methods [30]. In Egypt, 27% of 122 total *P. aeruginosa* isolates were positive for the production of MBL based on the results of phenotypic screening for MBL. This was lower than the prevalence of MBL producers in previous Egyptian studies, but similar to our present results 31.3% [8,31]. Studies in several parts of the world have demonstrated various rates of MBL production by *P. aeruginosa*, including 38.3% in Brazil, 35% in Canada, and 62% in Greece [32-34]. Variation in the detection rates of MBLs reported previously can be attributed to several factors that include: geographical locations; adherence to infection prevention and control precautions; number of samples tested and methods used to detect MBLs.

Among several genes encoding for MBLs, the VIM gene in *P. aeruginosa* is the most common [35]. In our study, the prevalence of VIM gene was 20.1%; these strains were isolated only from patients hospitalized for long periods in the ICU of the burns hospital. They had been intubated and undergone antibiotic therapy, mainly a combination of carbapenems and aminoglycosides and/or fluoroclonalones. In addition, all of these patients were infected with MDR isolates and the majority (85.2%) of these isolates was recovered from wounds of burn patients who had skin grafting. Mathlouti., *et al*. reported that the dissemination of genetically unrelated *bla-VIM*$_{-2}$ *P. aeruginosa* isolates (19/24) in Tripoli and Benghazi, Libya, the authors revealed that clones belong to ST11 and to ST235 being the most frequent [5]. In North Africa, *bla-VIM*$_{-2}$ was the most prevalent gene in MBL producing *P. aeruginosa* [8,36,37]. There are several reports on the prevalence of *bla-VIM* and *bla*$_{IMP}$ among carbapenem resistant *P. aeruginosa* strains isolates from burn patients in Iran [38,39]. The prevalence of carbapenemases is variable across the Middle East countries; this might be attributed to cross border transfer of patients as seen with injured Libyan combatant during the uprising, selective pressure from antibiotics, refugees and lack of compliance to standard precautions [40].

Conclusions

This study demonstrated that the majority of *P. aeruginosa* strains were resistant to various classes of antibiotics. The high incidence of antibiotic resistance among *P. aeruginosa* VIM producers is very alarming and can be responsible for serious infections especially among burn patients. Guidelines and appropriate infection prevention and control measures are needed to reduce these infections among patients. Further studies are needed to specify the most important genes of resistance among *P. aeruginosa* in Libya.

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Competing Interests

The authors declare that they have no competing interests.

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