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ORIGINAL PAPER

Metallo-Beta-Lactamase Producing *Pseudomonas aeruginosa* in a Healthcare Setting in Alexandria, Egypt

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Abstract

Pseudomonas aeruginosa has emerged as a major healthcare associated pathogen that creates a serious public health disaster in both developing and developed countries. In this work we aimed at studying the occurrence of metallo-beta-lactamase (MBL) producing *P. aeruginosa* in a healthcare setting in Alexandria, Egypt. This cross sectional study included 1583 clinical samples that were collected from patients admitted to Alexandria University Students' Hospital. *P. aeruginosa* isolates were identified using standard microbiological methods and were tested for their antimicrobial susceptibility patterns using single disc diffusion method according to the Clinical and Laboratory Standards Institute recommendations. Thirty *P. aeruginosa* isolates were randomly selected and tested for their MBL production by both phenotypic and genotypic methods. Diagnostic Epsilometer test was done to detect metallo-beta-lactamase enzyme producers and polymerase chain reaction test was done to detect imipenemase (IMP), Verona integron-encoded (VIM) and Sao Paulo metallo-beta-lactamase (IMP) encoding genes. Of the 1583 clinical samples, 175 (11.3%) *P. aeruginosa* isolates were identified. All the 30 (100%) selected *P. aeruginosa* isolates that were tested for MBL production by Epsilometer test were found to be positive; where 19 (63.3%) revealed bla_{SPM} gene and 11 (36.7%) had bla_{IMP} gene. bla_{VIM} gene was not detected in any of the tested isolates. Isolates of MBL producing *P. aeruginosa* were highly susceptible to polymyxin B 26 (86.7%) and highly resistant to amikacin 26 (86.7%). MBL producers were detected phenotypically by Epsilometer test in both carbapenem susceptible and resistant *P. aeruginosa* isolates. bla_{SPM} was the most commonly detected MBL gene in *P. aeruginosa* isolates.

Key words: *Pseudomonas aeruginosa*, Epsilometer test, metallo-beta-lactamases, MBL encoding genes

Introduction

Pseudomonas aeruginosa is considered one of the most leading causes of healthcare associated infections (HCAIs) worldwide (Varaiya *et al*., 2008). It is considered the fourth most commonly isolated nosocomial pathogen accounting for 10% of all HCAIs. *P. aeruginosa* infections can range from superficial skin infections to fulminant sepsis. Even colonization of such strains in critical systems can be fatal (Sivaraj *et al*., 2012).

World Health Organization (2015) has identified antimicrobial resistance as one of the three most important problems for human health. *P. aeruginosa* represents a phenomenon of resistance since all known mechanisms of antimicrobial resistance can be encountered; nevertheless enzyme production is the major mechanism of acquired resistance in these strains especially with β-lactam antibiotics, which are considerd a major line of treatment for *P. areuginosa*. Of these enzymes are the β-lactamases (Strateva and Yordanov, 2009). There are four classes of β-lactamases: A, C and D which act through a serine based mechanism and metallo-beta-lactamases (MBL); a class B type of β-lactamases that is the most worrisome and require bivalent metal ions, usually zinc as a cofactor for their activity (Bush and Jacopy, 2010). This group can be suppressed by bivalent ionic chelators as ethylene diamine tetra acetic acid (EDTA), but not inhibited by commercial β-lactamase inhibitors as clavulanic acid and tazobactam. They can hydrolyze β-lactams from all classes except the monobactams (Aoki *et al*., 2010).

MBL producing *P. aeruginosa* isolates were first reported in Japan in 1991, and since then there has been a substantial increase in the reporting of MBLs among carbapenem-resistant *P. aeruginosa* isolates worldwide (Pitout *et al*., 2005). These isolates increasingly have been responsible for several nosocomial outbreaks in tertiary centers in different parts of the world (Walsh, 2008). Also the association between infections caused by MBL producing *P. aeruginosa* and longer hospital stay with high mortality rates has been reported (Zavascki *et al*., 2006).

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The problem is aggravated by the fact that most of the MBL encoding genes reside on integrons and plasmids which in turn allows for widespread dissemination of these genetic elements, hence poses a threat for spread resistance patterns among the Gram-negative bacteria (Mohamed and Raafat, 2011).

As regards molecular structure: five MBL types have been widely recognized; imipenemase (IMP), Verona integron-encoded (VIM), Sao Paulo (SPM), German imipenemase (GIM) and Seoul imipenemase (SIM). Several types of MBL enzymes have been identified in *P. aeruginosa* among which the $_{VIM}$-type enzymes appear to be the most prevalent. IMP is also considered one of the most important types of MBLs. Camagaro *et al*., (2011) reported that after being restricted for more than ten years to Brazilian hospitals; SPM seems to become a global challenge, warning for the role of human traffic in spreading MBL genes (Salabi *et al*., 2010).

It is well known that poor outcome occurs when patients with serious infections due to MBL producing organisms are treated with antibiotics to which the organism is completely resistant. Therefore early and proper detection of MBL producing Gram-negative bacilli especially *P. aeruginosa* is crucial; for optimal treatment of particularly critically ill and hospitalized patients and to permit rapid initiation of strict infection control procedures to prevent nosocomial spread and control the dissemination of resistance (Cuzon *et al*., 2012). This work aimed at studying the occurrence of MBL producing *P. aeruginosa* in a healthcare setting in Alexandria, Egypt.

Experimental

Material and Methods

This cross sectional study was carried out during an 18-month period from January 2013 to June 2014. It included different clinical samples that were collected from patients admitted to the Alexandria University Students' Hospital (AUSH). Collected clinical samples were processed in AUSH laboratory and the Microbiology laboratory at the High Institute of Public Health (HIPH). The study was approved by the Ethics Committee of the HIPH. Informed consents of all enrolled patients were obtained before collection of samples and after explanation of the purpose and benefits of the research.

Sampling

Data collection. A questionnaire sheet including all the relevant information (name, age, sex, date of admission, medical history, diagnosis, antibiotic administration *etc*.) was filled in for every patient enrolled in the present study.

Samples collection and processing. A total of 1583 different clinical samples were collected during the study period from patients showing signs and symptoms suggestive of infection and were delivered to the laboratory. The samples were distributed as 660 respiratory samples (500 bronchoalveolar lavage (BAL) and 160 sputum samples), 446 urine samples, 209 blood samples, 142 pus and exudate samples, 58 peritoneal fluid samples, 35 ear discharge, and 33 conjunctival secretions. Collected samples were subjected to macroscopical and microscopical examination. The samples were cultured on blood (Oxoid 9191118 UK) and MacConkey's agar (Oxoid 567362 UK) plates. Plates were incubated aerobically at 37°C for 24 hours (Tille *et al*., 2014).

Identification procedures of *P. aeruginosa*. After proper incubation of inoculated blood and MacConkey's agar plates, isolates that appeared as medium sized, grayish, opaque, large flat pigmented colonies, with feathered edges, producing a sweet or grape like odour either hemolytic or non hemolytic on blood agar plates, and were pale, non lactose fermenting on MacConkey's agar plates, and microscopically appeared as Gram-negative bacilli were further differentiated and identified according to standard microbiological methods (Tille *et al*., 2014).

Antimicrobial susceptibility testing (AST). All 175 confirmed *P. aeruginosa* isolates were tested for their antibiotic susceptibility patterns using single disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (Patel *et al*., 2014). The test was done on Mueller Hinton (MH) agar plates (Difco 00252-01), using the selected antibiotic discs. All antimicrobial discs used in this study were supplied by oxoid laboratories. After 24 hours aerobic incubation at 37°C, each plate was examined and inhibition zones were measured, recorded, and interpreted as susceptible (S), intermediate (I) or resistant (R) according to the interpretive criteria of CLSI (Patel *et al*., 2014).

Isolates that were confirmed to be *P. aeruginosa* and tested for their antimicrobial susceptibility patterns were then subcultured on blood agar plates and incubated aerobically at 37°C for 24 hrs. Isolated colonies were inoculated on soft agar deeps and incubated aerobically at 37°C for 24 hrs.

Identification of metallo beta lactamase production by Epsilometer test (E-test). Thirty confirmed *P. aeruginosa* isolates were selected to be tested for their MBL enzyme production by E test. MBL diagnostic E-test strip consists of a double sided seven dilution range of imipenem (IP) (4 to 256 microgram/ml) and IP overlaid with EDTA (1 to 64 microgram/ml) (Pitout *et al*., 2005). MBL E-test was performed according to the manufacturer's instructions (AB BioMerieux,

Solna, Sweden). Isolates stored in agar deeps were subcultured on blood agar plates and were incubated aerobically at 37°C for 24 hrs. Individual colonies were picked from overnight agar plates and suspended in a 0.85% saline and were adjusted to a turbidity of 0.5 McFarland standard.

E-test MBL strips were applied to MH agar plates inoculated with adjusted suspensions. Seeded MH agar plates were incubated aerobically for 24 hrs at 37°C.The minimum inhibitory concentration (MIC) end points were read where the inhibition ellipses intersected the strip. A reduction of imipenem MIC in the presence of EDTA that is greater than or equal to eight-fold (IP/IPI > 8 mm) was interpreted as indicating MBL activity. The presence of a phantom zone or a deformation of the imipenem ellipse was also considered a positive result (Pitout *et al*., 2005).

Detection of bla_{IMP}, bla_{VIM}, and bla_{SPM} MBL genes by PCR. Thirty *P. aeruginosa* isolates that were confirmed as MBL producers by MBL diagnostic E-test, were tested for the presence of bla_{IMP}, bla_{VIM}, bla_{SPM} genes.

DNA extraction

Procedure. DNA for PCR was extracted by the boiling method. Two or three colonies were taken from fresh culture of the confirmed MBL *P. aeruginosa* isolates and suspended in 500 µl saline, then vortexed to get a uniform suspension. The cells were lysed by heating them at 100°C for 10 minutes, and then centrifuged at 12,000 rpm for 10 min. The supernatant was used directly as a template DNA in the PCR mixture.

DNA amplification. The extracted DNA was subjected to PCR amplification reaction using three pairs of primers specific for MBL genes (bla_{IMP}, bla_{VIM}, and bla_{SPM}). The DNA amplification was done using Dream Taq Green PCR Master mix (Thermo Scientific, Waltham, United States). The primers were purchased lyophilized; (Biosearch Tech, Petaluma, California, United States). They were reconstituted by the addition of sterile nuclease free water to a final concentration of 100 pico mol/µl, distributed in aliquots and stored at –20°C.

Primers sequence of MBL genes (Sader *et al*., 2005)

Primer	Sequence (5' to 3')
IMP 1	Sense: 5'dCTACCGCAGCAGAGTCTTTGC3' Antisense: 5'dGAACAACCAGTTTTGCCTTACC3'
VIM 2	Sense: 5'dATGTTCAAACTTTTGAGTAGTAAG3' Antisense: 5'dCTACTCAACGACTGAGCG3'
SPM 1	Sense: 5dCCTACAATCTAACGGCGACC3' Antisense: 5'dTCGCCGTGTCCAGGTATAAC3'

b-PCR amplification protocol. (I) Reaction mixtures were prepared using sterile nuclease free water. To each tube a total volume of 50 ml was reached by adding Master mix (25 µl), sense primer (1 µl), antisense primer (1 µl), DNA template (sample) (10 µl), nuclease free water (13 µl). A negative control was prepared by the addition of the same contents to the tube with 10 µl nuclease free water instead of the sample.

(II) The tubes were transferred to the thermal cycler (BioCycler TC-S, Boeco-Germany) for amplification. The thermocycler program conditions for bla_{IMP} and bla_{VIM} genes included: 30 cycles of amplification under the following conditions: denaturation at 95°C for 30 seconds, annealing for 1 minute at specific temperatures (bla_{IMP} at 45°C and bla_{VIM}–66°C), and extension at 72°C for 1 minutes/kb product (Khosravi *et al*., 2011). The cycling parameters of PCR to amplify bla_{SPM} gene were: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 minute and extension at 68°C for 1 minute. The cycle was followed by a final extension at 72°C for 10 minutes (Gaspareto *et al*., 2007).

DNA detection by gel electrophoresis. PCR products were loaded on 2% agarose in tris borate EDTA (TBE) containing 0.5 µl of ethidium bromide per ml. After electrophoresis, the gel was visualized under ultraviolet light.

The DNA bands were visualized on a 320 nm UV transilluminator and photographed. The gel was examined for specific bands; positive results of PCR were confirmed by detection of 432 bp band for bla_{IMP} gene, 500 bp band for bla_{VIM} gene and 650 bp band for bla_{SPM} gene as determined by the molecular weight markers run at the same time. (Khosravi *et al*., 2011; Gaspareto *et al*., 2007).

Statistical analysis of the data. Data were fed to the computer and analyzed using IBM SPSS software package version 20.0 (Kirkpatrick and Feeney, 2013). Qualitative data were described using number and percent. Quantitative data were described using Range (minimum and maximum), mean, standard deviation and median. Comparison between different groups regarding categorical variables was tested using Chi-square test, Fisher's exact test, Monte Carlo correction, independent t-test and Mann Whitney test. Significance of the obtained results was judged at the 5% level.

Results

The present study included 175 *P. aeruginosa* isolates that were recovered from a total of 1583 clinical samples. These samples were collected randomly from patients admitted to the AUSH (984 from ICUs and 599 from wards).

The highest percentage of *P. aeruginosa* isolates 75 (42.9%) were recovered from respiratory samples, followed by urine samples 57 (32.6%) and pus and exudate

samples 33 (18.9%). From blood samples, four *P. aeruginosa* isolates were recovered (2.3%), while each of conjunctival secretion, ear discharge, and peritoneal fluid samples yielded two *P. aeruginosa* isolates (1.1% each).

In the current work, the highest percentage of resistance among 175 detected *P. aeruginosa* isolates was for aztreonam 150 (85.7%), followed by ceftazidime 140 (80%), cefipime 139 (79.4%), imipenem 137 (78.3%), ciprofloxacin 134 (76.6%), and meropenem 129 (73.7%).

On the other hand, the highest susceptibility percentages were recorded for polymyxin B 152 (86.9%), piperacillin tazobactam 52 (29.7%), 48 ofloxacin (27.4%), piperacillin 46 (26.3 %), gentamycin 39 (22.3%) and cefepime 28 (16%).

Of the 175 confirmed *P. aeruginosa* isolates, 30 isolates were randomly selected to be tested phenotypically for their MBL enzyme production by E test. Thirty selected *P. aeruginosa* isolates were as follows:

- Twenty *P. aeruginosa* isolates were resistant to three classes of β-lactams including: cephems, carbapenems and monobactams (aztreonam).
- Three *P. aeruginosa* isolates were resistant to three classes of β-lactams (penicillins, cephalosporins and carbapenems) and were susceptible to aztreonam.
- Seven *P. aeruginosa* isolates were resistant to three classes of antibiotics (cephems, aminoglycosides, and carbapenems), but were susceptible to imipenem, and 4 of them were also susceptible to meropenem.

This study showed that the highest percentage of MBL producing *P. aeruginosa* isolates were from respiratory samples 11/30 (36.7%), followed by urine, and pus and exudate samples 9/30 (30%) each. Only one isolate was recovered from an ear sample 1/30 (3.3%) (Fig. 1).

Thirty selected isolates were all positive for MBL production by MBL E test, and were tested for bla_{IMP}, bla_{VIM} and bla_{SPM} genes using conventional PCR (results as in Fig. 2). Of the 30 tested isolates; 19 (63.3%)

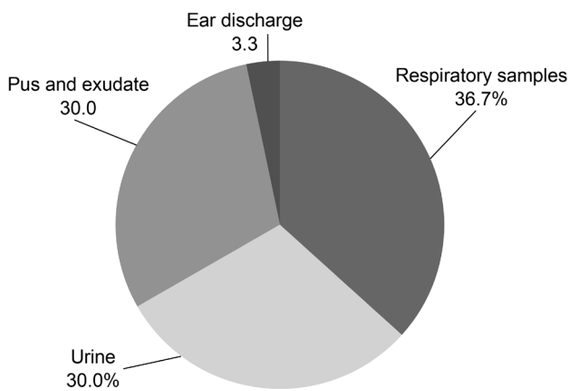

Fig. 1. Distribution of 30 MBL producing *P. aeruginosa* isolates according to type of samples.

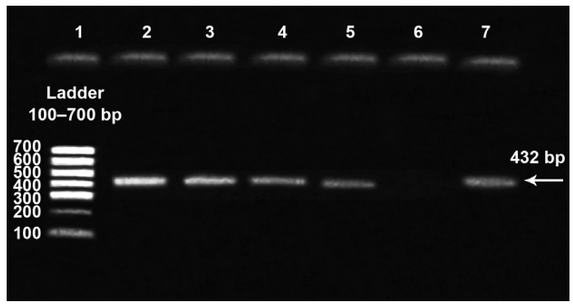

Fig. 2A bla_{IMP}
Lane 1: Marker 100 bp Ladder
Lane 2: Positive test strain for bla_{IMP} (432 bp)
Lane 3: Positive test strain for bla_{IMP} (432 bp)
Lane 4: Positive test strain for bla_{IMP} (432 bp)
Lane 5: Positive test strain for bla_{IMP} (432 bp)
Lane 6: Negative control
Lane 7: Positive test strain for bla_{IMP} (432 bp)

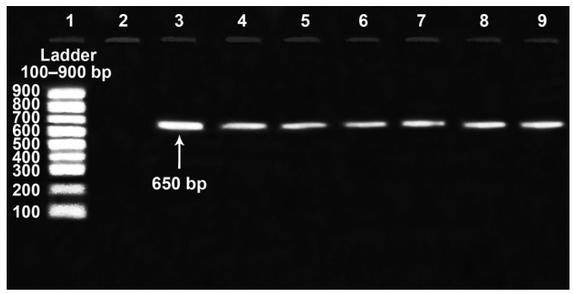

Fig. 2B bla_{SPM}
Lane 1: Marker 100 bp Ladder
Lane 2: Negative control
Lane 3: Positive test strain for bla_{SPM} (650 bp)
Lane 4: Positive test strain for bla_{SPM} (650 bp)
Lane 5: Positive test strain for bla_{SPM} (650 bp)
Lane 6: Positive test strain for bla_{SPM} (650 bp)
Lane 7: Positive test strain for bla_{SPM} (650 bp)
Lane 8: Positive test strain for bla_{SPM} (650 bp)
Lane 9: Positive test strain for bla_{SPM} (650 bp)

Fig. 2C bla_{VIM}
Lane 1: Marker 100 bp Ladder
Lane 2: Negative test strain for bla_{VIM} (500 bp)
Lane 3: Negative test strain for bla_{VIM} (500 bp)
Lane 4: Negative control
Lane 5: Negative test strain for bla_{VIM} (500 bp)
Lane 6: Negative test strain for bla_{VIM} (500 bp)
Lane 7: Negative test strain for bla_{VIM} (500 bp)
Lane 8: Negative test strain for bla_{VIM} (500 bp)

Fig. 2. A, B, C. Electrophoresis results for *P. aeruginosa* bla_{IMP}, bla_{SPM} and bla_{VIM} genes.

Table I
Distribution of the 30 patients with MBL producing *P. aeruginosa* isolates according to their gender and age.

Gender / Age in years	Male (n = 12)		Female (n = 18)		Total (n = 30)	
	No.	%	No.	%	No.	%
20– < 30	7	58.3	7	38.9	**14**	**46.7**
30–50	1	8.3	8	44.4	**9**	**30.0**
> 50	4	33.3	3	16.7	**7**	**23.3**
χ^2 (^{MC}p)	4.570 (0.104)					

^{2}: Chi square test; MC: Monte Carlo test

revealed bla_{SPM} gene and 11(36.7%) had bla_{IMP} gene, bla_{VIM} gene was not detected in any of the tested isolates.

Of the 30 patients with MBL producing *P. aeruginosa* isolates, 14(46.7%) were of age group 20 ≤ 30 years, 9 (30.0%) belonged to the age group 30–50 years, and seven patients (23.3%) were above 50 years (Table I). Moreover, our data showed that the highest percentage of patients who revealed *P. aeruginosa* isolates with positive bla_{SPM} gene were aged 20 ≤ 30 years old (47.4%), followed by those aged > 50 years old (31.6%).

In this study the majority of patients with MBL producing *P. aeruginosa* isolates 21 (70%) were admitted to the ICU and 9 (30%) were admitted in inpatient wards. In addition, the highest percentage (76.7%) was recovered from those who had duration of hospital stay of more than 7 days. Regarding antibiotic intake, the majority of patients 26/30 (86.7%) had taken antibiotics within 2 weeks from the study period, and this was found to be statistically significant (p ≤ 0.001). On the other hand, 23/30 (76.7%) patients were readmitted to the hospital within 3 months. This was statistically significant (p ≤ 0.006) (Table II).

Our work revealed that the 30 MBL producing *P. aeruginosa* isolates were highly susceptible to polymxin B 26 (86.7%), followed by piperacillin-tazobactam 11 (36.7%), then gentamycin 8 (26.7%). Seven (23.3%) isolates were susceptible to each of imipenem and ofloxacin. The highest percentage of isolates were resistant to Amikacin 26/30 (86.7%), followed by piperacillin and ciprofloxacin 24 /30(80% each) (Table III).

More than half of the patients who had *P. aeruginosa* isolates with bla_{IMP} genes 6/11 (54.5%) were aged between 20 ≤ 30 years old, this was followed by those of 30–50 years old 3/11 (27.3%). The lowest percentage of *P. aeruginosa* isolates with bla_{IMP} gene 2 (18.2%) was recovered from patients above 50 years. On the other hand, the highest percentage of patients who revealed positive bla_{SPM} gene were aged 20 ≤ 30 years old 9/19 (47.4%), followed by those aged > 50 years old 6/19 (31.6%).

Table II
Distribution of 30 patients with positive MBL producing *P. aeruginosa* isolates according to risk factors.

Frequency of isolation / Risk factors	Patients with MBL producing *P. aeruginosa* isolates (n = 30)		Test of sig.	P
	No.	%		
Site of admission				
ICU (n = 126)	21	70.0	$\chi^2 = 0.072$	0.789
Ward (n = 49)	9	30.0		
Length of hospital stay (days)				
≤ 7 (n = 54)	7	23.3	$\chi^2 = 0.961$	0.327
> 7 (n = 121)	23	76.7		
Min. – Max.	3.0–39.0		Z = 0.519	0.604
Mean ±SD.	14.97 ± 10.12			
Median	12.50			
Associated diseases				
DM	22	73.3	$\chi^2 = 18.114$*	< 0.001*
Cancer	18	60.0	$\chi^2 = 9.052$*	0.003*
Related devices				
Mechanical ventilator	7	23.3	$\chi^2 = 0.784$	0.376
Urinary catheter	7	23.3	$\chi^2 = 8.823$*	^{FE}p = 0.024*
Readmission (n = 94)	23	76.7	$\chi^2 = 7.672$*	0.006*
Antibiotic intake (n = 97)	26	86.7	$\chi^2 = 14.301$*	< 0.001*

χ^2: Chi square test; Z: Z for Mann Whitney test; *: Statistically significant at p ≤ 0.05

Table III
Antimicrobial susceptibility patterns of 30 MBL producing *P. aeruginosa* isolates.

Susceptibility pattern / Antimicrobial agent	Susceptible S		Intermediate I		Resistant R	
	No	%	No	%	No	%
Penicillins						
– Piperacillin	4	13.30	2	6.7	24	80
β-lactam/β-lactamase inhibitor combinations						
– Piperacillin – tazobactam	11	36.7	8	26.7	11	70
Cephems						
– Ceftazidime	2	6.7	10	33.3	18	60
– Cefepime	5	16.7	4	13.3	21	70
Carbapenems						
– Imipenem	7	23.3	1	2.7	22	73.3
– Meropenem	4	13.3	6	20	20	66.7
Monobactams						
– Aztreonam	3	10	5	16.7	22	73.3
Aminoglycosides						
– Gentamycin	8	26.7	2	6.7	20	66.7
– Amikacin	1	3.3	3	10	26	86.7
Fluoroquinolones						
– Ciprofloxacin	5	16.7	1	33.3	24	80
– Ofloxacin	7	23.3	2	6.7	21	70
Lipopeptides						
– Polymyxin B	26	86.7	0	0.0	4	13.3

Table IV
Relation between results of blaIMP gene among 30 patients with MBL producing *P. aeruginosa* isolates and their risk factors.

Risk factors	bla_{IMP} gene				Test of Sig.	P
	Negative (n = 19)		Positive (n = 11)			
	No.	%	No.	%		
Site of admission						
ICU	13	68.4	8	72.7	$\chi^2 = 0.062$	$^{FE}p = 1.000$
Ward	6	31.6	3	27.3		
Length of hospital stay (days)						
≤ 7	5	26.3	2	18.2	$\chi^2 = 0.258$	$^{FE}p = 0.686$
> 7	14	73.7	9	81.8		
Min. – Max.	4.0 – 37.0		3.0 – 39.0		Z = 0.129	0.897
Mean ± SD.	14.68 ± 9.84		15.45 ± 11.05			
Median	12.0		14.0			
Associated diseases						
DM	15	78.9	7	63.6	0.835	0.417
Cancer	11	57.9	7	63.6	0.096	1.000
Related devices						
Mechanical ventilator	4	21.1	3	27.3	0.151	1.000
Urinary catheter	4	21.1	3	27.3	0.151	1.000
Hospital readmission	7	36.7	9	81.8	5.662*	0.017*
Antibiotic intake	10	52.6	8	72.7	1.172	0.442

χ^2: value for Chi square; MC: Monte Carlo test
FE: Fisher Exact test Z: Z for Mann Whitney test *: Statistically significant at $p \leq 0.05$

Table V
Relation between results of blaSPM gene among 30 patients with MBL producing *P. aeruginosa* isolates and their risk factors.

Risk factors	bla_{SPM} gene				Test of Sig.	P
	Negative (n = 11)		Positive (n = 19)			
	No.	%	No.	%		
Site of admission						
ICU	7	63.6	14	73.7	$\chi^2 = 0.335$	$^{FE}p = 0.687$
Ward	4	36.4	5	26.3		
Length of hospital Stay(days)						
≤ 7	2	18.2	5	26.3	$\chi^2 = 0.258$	$^{FE}p = 0.686$
> 7	9	81.8	14	73.7		
Min. – Max.	3.0 – 37.0		4.0 – 39.0		Z = 0.539	0.590
Mean ± SD.	14.18 ± 11.03		15.42 ± 9.84			
Median	11.0		14.0			
Associated diseases						
DM	1	9.0	5	26.3	3.135	$^{FE}p = 0.104$
Cancer	0	0.0	7	36.8	1.408	0.235
Related devices						
Mechanical ventilator	0	0.0	7	36.8	5.286*	$^{FE}p = 0.029$*
Urinary catheters	2	18.2	5	26.3	0.740	$^{FE}p = 0.658$
Hospital readmission	4	36.4	15	78.9	5.440*	$^{FE}p = 0.047$*
Antibiotic intake	3	27.3	13	68.4	4.739*	0.029*

χ^2: value for Chi square; MC: Monte Carlo test
FE: Fisher Exact test Z: Z for Mann Whitney test *: Statistically significant at p ≤ 0.05

P. aeruginosa with positive bla_{IMP} gene were isolated from 7 patients with cancer and 7 with diabetes mellitus (DM) (63.6% each). In addition, 3 patients were on mechanical ventilators (27.3%) and 3 had urinary catheters (Table IV). On the other hand, *P. aeruginosa* isolates with bla_{SPM} gene were recovered from 5 (26.3.2%) patients, who had DM and 7 (36.8%) who suffered from cancer (Table V).

Discussion

P. aeruginosa has emerged as a major HCA pathogen in both developing and developed countries. This organism creates a serious public health disaster resulting in an enormous burden of morbidity, mortality and high health care cost, especially among high risk patients in ICUs (Morales *et al*., 2012).

In the present study, 175 (11.1%) *P. aeruginosa* isolates were recovered from a total of 1583 clinical samples that were collected from patients admitted to the AUSH (984 were admitted to ICUs and 599 wards). A slightly higher percentage was reported by Divyashanthi *et al.* (2015), where 15.2% *P. aeruginosa* isolates were recovered from 788 tested clinical samples. In Nepal, Khan *et al.* (2014), reported that 194 (21.1%) *P. aeruginosa* isolates were identified from 917 collected clinical samples from patients with suspected *P. aeruginosa* infections. A much higher isolation rate (41.5%) was recorded by Sedighi *et al.* (2012), in Iran, where 106 *P. aeruginosa* isolates were detected from 255 gathered clinical samples. On the other hand, lower percentages of isolation were reported in a 3-year study that was conducted by Mohanasoundaram (2011) to determine the distribution rate and antimicrobial resistance pattern in *P. aeruginosa* among clinical samples (5%, 6.8% and 5% in 2008, 2009 and 2010, respectively).

One of the main concerns about *P. aeruginosa* is its remarkable ability to rapidly develop antibiotic resistance. The wide array of antimicrobial resistance mechanisms that have been described for *P. aeruginosa* is impressive and rivals those of other non-fermentative Gram-negative pathogens and illustrates the potential of this organism to respond swiftly to changes in selective environmental pressure. In recent years, Egypt has been considered among the countries that reported high rates of antimicrobial resistance (Zafer *et al*., 2014)

In the current study, the susceptibility patterns of *P. aeruginosa* isolates were tested by a panel of antimicrobial agents according to CLSI recommendations. It was found that *P. aeruginosa* isolates showed high levels of resistance to many antibiotics. One of the

alarming results is the high resistance against carbapenems; where 78.3% of isolates were resistant to imipenem and 73.7% were resistant to meropenem. In agreement with our results, Diab *et al.* (2013) revealed a high rate (72%) of imipenem resistance among *P. aeruginosa* isolates. In the Middle East the occurrence of imipenem resistant *P. aeruginosa* was also recognized. In Saudi Arabia, of a total of 350 *P. aeruginosa* isolates, 135 (38.57%) were found to be resistant to imipenem (Mohamed *et al.*, 2011). Hashemi *et al.*, (2016) reported that all their MBL-producing *P. aeruginosa* isolates were resistant to meropenem and imipenem.

Among 33 European countries participating in the European Antimicrobial Resistance Surveillance System (EARSS) in 2007, six countries reported carbapenem resistance rates of more than 25% among *P. aeruginosa* isolates (Souli *et al.*, 2008); the highest rate was reported from Greece (51%). Reasons for increased antimicrobial resistance in Greece are numerous as explained by Miyakis *et al.* (2011). As Greece has the highest antibiotic consumption rate in Europe, both in total and in out-patients, population mobility can introduce resistant strains and infrastructure and resources for infection control are insufficient. This, along with reduced awareness for detection, increases the likelihood of in-hospital spread of multi drug resistant organisms (MDROs). Many of these reasons exist in our country and can explain the high antimicrobial resistance encountered among *P. aeruginosa* isolates in the current study.

During the last decade, emergence and dissemination of the most prevalent MBL genes such as; bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM}, and the newly identified bla_{AIM} and bla_{NDM} have been extensively documented around the world. Because of the efficient carbapenemase activity of the MBL enzymes, they account for up to 40% of worldwide imipenem resistant *P. aeruginosa.* Xavier *et al.* (2010) stated that MBL enzymes increase antimicrobial MICs more effectively than does either efflux pump over-expression or porin down-regulation alone. Unfortunately, screening only carbapenem-resistant organisms for these enzymes, as most often performed, is suboptimal. With increasing reports of MBLs in carbapenem-susceptible isolates, it becomes crucial to improve laboratory methods used for detection of what is called: hidden MBLs (Ntokou *et al.*, 2008).

In the present study determination of MBL frequency in both carbapenem resistant and susceptible *P. aeruginosa* isolates was done by phenotypic and genotypic methods. Accordingly, 30 identified *P. aeruginosa* isolates were selected to cover: the commonly known definition of MBL producing *P. aeruginosa* isolates *i.e.* those that are resistant to all beta lactams but sensitive to monobactams (aztreonam), and isolates that showed resistance to carbapenems and aztreonam, together with isolates that were carbapenem (imipenem) sensitive to exclude hidden MBLs.

The most widely accepted standardized MBL screening test is the MBL E-test. However, due to the high cost and relative unavailability of E-test strips, many clinical microbiology laboratories use alternative screening methods, such as double-disk synergy test (DDST) and combined disk test (CDT). Although the DDST and the CDT assays are simple to perform and cheaper than the MBL E-test, they have shown discordant results, depending on the employed methodology, β-lactam substrates, MBL inhibitors, and bacterial genus tested (Picão *et al.*, 2008; Ranjan *et al.*, 2015). In this piece of work, all of the 30 (100%) selected *P. aeruginosa* isolates were found to be MBL producing strains using E-test. Similarly Bashir *et al.* (2011) reported that all their thirty isolates were positive for MBL producing *P. aeruginosa* by E-test.

In the current work, of the 30 identified MBL isolates; the highest percentage was recovered from respiratory samples 11 (36.7%), followed by 9 (30%) from each of urine and pus and exudate samples, and only one isolate (3.3%) was from ear discharge. This is nearly in agreement with Zavascki *et al.* (2006), who found that the lung was the most frequent site of nosocomial infection (50.3%), followed by urinary tract (20.5%), and skin and soft tissue (15.8%).

Many factors may contribute to the acquisition of MBL resistant enzymes. Patients in critical care units are likely to have higher probability for these isolates. It was found from this study that the highest percentage of MBL producing *P. aeruginosa* isolates was among ICU patients (70%). This was in line with Zavascki *et al.* (2006) and Lucena *et al.* (2014) who found that ICUs admission increased the risk for MBL producing *P. aeruginosa* infections.

One of the most important parameters for MBL acquisition is associated diseases. Of the 30 patients, who revealed MBL *P. aeruginosa* isolates, 22 (73.3%) had DM and 18 (60%) suffered from cancer. These results were statistically significant ($p < 0.001$ and $p = 0.003$, respectively). In concordance, Varaiya *et al.* (2008) reported that out of 33 (14.3%) MBL producing isolates, 24 (72.7%) were diabetic patients, and 6 (18.1%) were cancer patients. They attributed their findings to the associated immune deficiency among diabetic patients, and recurrent foot infections being good poly-microbial media for a high incidence of multidrug-resistant *P. aeruginosa*. In addition, Vaishali *et al.* (2013) reported that the presence of underlying diseases as DM is a significant risk factor in acquisition of MBL *P. aeruginosa* infection.

Other risk factors that were significantly associated with the presence of MBL producing *P. aeruginosa* infection in this study were the use of medical devices

as mechanical ventilators and urinary catheters (23.3%) each, readmission to hospitals (76.7%), and history of previous antibiotic intake (86.7%). On the other hand, the length of hospital stay for more than 7 days represented 76.7%, but was not found to be statistically significant. Kumar *et al.* (2011), found that all MBL-positive patients were exposed to different risk factors as prolonged hospital stay for more than 8 days, catheterization, previous antibiotic use, and mechanical ventilation. In the study done by Vaishali *et al.* (2013) the duration of hospital stay of more than 10 days had 1.7 times risk of acquisition of MBL *P. aeruginosa* infection more than duration of hospital stay of less than or equal to10 days.

The occurrence MBL production is not restricted to carbapenem resistant strains, but some reports have argued about their presence in carbapenem susceptible organisms. They might be unrecognized as the MBL detection has not been routinely performed in most clinical microbiology laboratories (Picão *et al.*, 2012). Such organisms often carry hidden MBL genes. As a consequence, these isolates will be able to participate in horizontal MBL gene transfer with other Gram-negative pathogens and may contribute significantly to MBL related outbreaks. In the present study, of the 30 identified MBL producing *P. aeruginosa* isolates, 7 (23.3%) were imipenem sensitive and 4 (13.3%) were sensitive to meropenem. Diab *et al.* (2013) documented that among the studied imipenem susceptible isolates, 58.3% of which were proved to be MBL producers. A much lower percentage was published by Anoar *et al.* (2014) in Iraq, where of the 46 detected MBL producing isolates, 7 (3.95%) were meropenem sensitive. They explained their findings by the possibility that there might be hidden MBL genes among isolated strains which cannot be diagnosed by phenotypic tests, leading to the dissemination of these genes in the hospital silently among patients even within normal HCWs who can act as carriers for MBL genes in future.

In this piece of work, multi drug resistance was noticed. MBL producing *P. aeruginosa* had the highest percentage of resistance against amikacin 86.7%, followed by piperacillin and ciprofloxacin 80% each. In addition, carbapenems showed high percentages of resistance; imipenem and meropenem (73.3%, and 66.7%, respectively). Bhongle *et al.* (2012) found only one isolate sensitive to imipenem, but it was found to be positive for MBL, thus indicating that MBL producers could show susceptibility to imipenem. These isolates can appear to be susceptible to carbapenems though they carry carbapenemases, such organisms thus carry hidden MBL genes, whereby the microbiologists may remain unaware of their presence.

MBL producers are commonly known as those organisms that potently hydrolyze all beta-lactam antibiotics except aztreonam. In the current study, high resistance to monobactams (aztreonam) was detected (73.3%), and only 3 (10%) of the isolates were susceptible to aztreonam and conformed to such definition. A lower percentage of aztreonam resistance (45.1%) was reported by Zafer *et al.* (2014). In this study, 86.7% of MBL producing *P. aeruginosa* isolates were susceptible to polymyxin B. This supports the evidence that polymyxin B has increasingly become the last viable therapeutic option for MDR *Pseudomonas* infections. In accordance with the current results Bashir *et al.* (2011) recorded that MBL producers showed very high resistance to all antimicrobials except polymyxin B. However, resistance to amikacin (73%) and ciprofloxacin (55%) was lower when compared to the results in the present study (86.7% and 80%, respectively). Piperacillin tazobactam was the second most effective antibiotic after polymyxin B, where 36.7% of our isolates were sensitive to it.

Although phenotypic methods are considered to be useful and reliable in detecting MBL producers, results should be validated and confirmed by genotypic methods. In the present study, all the 30 (100%) identified MBL producing *P. aeruginosa* isolates by E test were further screened for the presence of MBL encoding genes (bla_{IMP}, bla_{SPM}, and bla_{VIM}) using conventional PCR. bla_{IMP} gene was the most prevalent MBL among the isolates accounting for 63.3%, while 36.7% of the isolates revealed bla_{IMP} gene and none of the isolates had bla_{VIM} genes.

In agreement with the results of this study, Gaspareto *et al.* (2007) stated that bla_{SPM} gene was the most common gene among MBL *P. aeruginosa* isolates (73%), and that no bla_{VIM} gene was detected. Camargo *et al.* (2011) reported that bla_{SPM} gene accounted for 71% of positive MBL strains, while bla_{IMP} was detected in 29%.

On the contrary, bla_{VIM} gene was detected in many Egyptian studies. Zafer *et al.* (2014) demonstrated that 58.3% were positive for bla_{VIM} gene, with bla_{IMP} gene detected in 2.1% and no bla_{SPM} gene was identified. Another study by Diab *et al.* (2013) revealed that 70% were positive for bla_{VIM}, while bla_{IMP} gene was not detected.

In the current work, all the 30 *P. aeruginosa* isolates that were identified as MBL producers by the phenotypic method E-test, were found to be MBL producers by the molecular method PCR; where 19 isolates had MBL_{SPM} gene and 11 had bla_{IMP} gene. A lower percentage was reported by Doosti *et al.* (2013); where 36/41 (87.8%) of isolates were phenotypically MBL positive, but PCR results confirmed presence of MBL genes in only 33/41 (80%) of isolates. bla_{IMP} producers have been detected worldwide: in Europe (Docquier *et al.*, 2003) and in the U.S. (Hanson *et al.*, 2006). In the current study, positive MBL IMP gene producers were more commonly

isolated from respiratory samples (36.4%), followed by urine and pus and exudate samples (27.3% each). It was detected in only one ear discharge sample (9.1%).

In the current work, hospital readmission was found to be a significant risk factor for acquiring bla_{IMP} gene, where 81.8% of patients were readmitted to the hospital within 3 months of the study ($p = 0.017$).

Among MBL genes, bla_{SPM} has been widely detected in Brazil and Switzerland, the dissemination of this gene in various regions seems to be caused by a single epidemic *P. aeruginosa* clone (Salabi *et al*., 2010) In the present study, bla_{SPM} gene producers were mostly recovered from respiratory samples (47.4%), followed by urine (26.3%) and pus and exudate samples (21.1%). In line to our data, Zavascki *et al*. (2006) in southern Brazil, described the first nosocomial outbreak of *P. aeruginosa* producing MBL SPM gene, and noted that $_{IMP}$ isolates were highly revealed from respiratory samples (43.0%), followed by urine and surgical wound samples (33.3% and 5.9%, respectively). While Matos *et al*. (2016) documented that their study was the first report describing the detection of the bla_{SPM}-1-like gene in northern Brazil.

In the present study, the majority of patients with positive bla_{SPM} gene *P. aeruginosa* isolates were admitted to the ICU (73.7%) and 14 (73.7%) had a hospital stay duration of more than 7 days. In addition 5 (26.3%) had DM and 7 (36.8%) suffered from cancer. It was also found that the use of mechanical ventilators was significantly associated with positive bla_{SPM} genes ($p = 0.029$). Other risk factors that were significantly associated with higher rates of positive bla_{SPM} gene were history of hospital readmission and antibiotic intake (78.9% and 68.4%, respectively). In agreement with the current findings, a study to evaluate risk factors for colonization or infection due to MDR *P. aeruginosa* carrying bla_{SPM} gene recorded that 50% of patients with positive bla_{SPM} gene had Foley's catheters, 21% were on mechanical ventilation, 78% had been previously hospitalized within the preceding year and all patients (100%) had taken antibiotics which was the main significant risk factor detected (Nouer *et al*., 2005). Matos *et al*. (2016) reported that 20% (4/20) of their *P. aeruginosa* isolates were positive for the bla_{SPM}-1-like gene, and that MDR occurred most frequently among isolates from adults who had been hospitalized for an average of 87.1 days, where the use of mechanical ventilation and urinary catheters were risk factors for infection.

In contrast with other studies (Zafer *et al*., 2014; Mohd *et al*., 2015) which suggested successful global dissemination of bla_{VIM} resistant gene and considered it to be of great concern, no bla_{VIM} genes were detected in the present work. Zafer *et al*., in 2014 reported that bla_{VIM} gene was detected in 85% of MBL producing *P. aeruginosa* isolates, while Gonçalves *et al*., (2017) reported that among the 157 analyzed *P. aeruginosa* strains, 5.3% were positive for bla_{VIM} gene.

Ignorance of rational antibiotics prescribing principles and prolonged clinical use of carbapenems for the treatment of MDR *P. aeruginosa* infections have been recognized as the main reasons behind MBL carbapenamase producing strains. Determination of MBL genes in MDR *P. aeruginosa* gives useful data about their epidemiology and risk factors associated with this group. Hence, early recognition of MBL producers is indispensable and necessitates rigorous infection control measures (Cantas *et al*., 2013).

Conclusions

- MBL producing *P. aeruginosa* isolates were more prevalent among patients admitted to the ICUs.
- Polymyxin B was the most effective antimicrobial agent against MBL producing *P. aeruginosa* isolates, while amikacin was the least effective one.
- Monobactam (aztreonam) susceptible *P. aeruginosa* isolates were also found to be MBL producers as aztreonam resistant ones.
- MBL producers were detected phenotypically by E test in both carbapenem susceptible and resistant *P. aeruginosa* isolates.
- bla_{SPM} was the most commonly detected MBL gene in *P. aeruginosa* isolates.
- bla_{SPM} and bla_{IMP} MBL encoding genes were detected in both carbapenem susceptible and resistant *P. aeruginosa* isolates.
- Associated diseases (DM, cancer), indwelling urinary catheters, hospital readmission, and antibiotic intake were considered as significant risk factors for MBL producing *P. aeruginosa* infections.

Recommendations

1. Accurate laboratory methods including culture and antimicrobial susceptibility testing with routine screening for MBL production should be the base for proper diagnosis and management of *P. aeruginosa* infections.
2. Early and reliable detection of MBL production in *P. aeruginosa* isolates including monobactam and carbapenem susceptible and resistant strains; to permit timely institution of effective antimicrobial therapy and control dissemination of resistance in hospitals.

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