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ORIGINAL ARTICLE

Detection of Metallo-β –lactamase–producing *Psudomonas aeruginosa* Strains Isolated from Nosocomial Infections in Suez Canal University Hospital, Ismailia, Egypt

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Key words:

ESBL, MBL, pseudomonas aeruginosa, blaIMP, blaVIM, Egypt

ABSTRACT

Background: *Pseudomonas aeruginosa causes large percentages of nosocomial infections with high rates of treatment failure due to antibiotic resistance. In recent years, metallo beta lactamase (MBL) resistance has emerged in pseudomonas aeruginosa isolates which belong to Ambler class B.* ***Objectives:*** *We have performed this study to find out the frequency of MBL- producing Pseudomonas aeruginosa.* ***Methodology:*** *This study was conducted on 120 clinical specimens isolated from Suez Canal University Hospital. P. aeruginosa were isolated and identified conventionally. All isolates were submitted for antibiotic susceptibility testing, followed by phenotypic screening tests for detection of ESBL production using cefotaxime disc (30µg) and ceftazidime disc (30µg) and then confirmed by using disc combination method of ceftazidime- clavulanic acid and cefotaxime- clavulanic acid discs. Double disc synergy test was performed. MBL was suspected when the isolate was resistant to meropenem and imipenem confirmed by Modified Hodge test (MHT) and EDTA disk synergy test. PCR targeting genes bla IMP and bla VIM was applied for more accurate detection.* ***Results:*** *Out of 120 clinical samples, 26 isolates were diagnosed as pseudomonas aeruginosa. All isolates were considered as potential producer of carbapenemases.The resistance rates were 42.3% for cefepime and reached 88.5% for ceftazidime. We found that 80.8% of the isolates were ESBL producers and 54.8% (14 isolates) were considered as class B carbapenemases (MBL) by using MHT and EDTA synergy test. PCR detected bla IMP and blaVIM in six and seven isolates, respectively. The relation between MBL genes and resistance pattern of the isolates to cephalosprines, Aztreonam and gentamicin was not statistically significant (p=0.9).* ***Conclusion:*** *ESBLs and MBLs are still playing a major role in marked antibiotic resistance of pseudomonas aeruginosa against the extended beta lactam antibiotics in Ismailia, Egypt. The current study provides a new report for detection of blaIMP and blaVIM in Ismailia, Egypt.*

INTRODUCTION

Pseudomonas aeruginosa is a common opportunistic pathogen responsible for many hospital-acquired infections [1]. The organism is widely distributed in nature throughout the world. It is a Gram negative, non sporing, non capsulated, straight or slightly curved rod shaped bacterium occurring singly, in pairs or in short chains [2]. It has the ability to live in disinfectants, respiratory equipment, sinks, taps, and mops within the hospital in the form of biofilm and can enter the hospital environment through visitors and patients or goods that enter in hospital [3]. Therefore, it is reported to be one of the most notorious organisms that cause hospital acquired infections.

Pseudomonas aeruginosa infections are hard to treat due to the natural resistance of the species, as well as to its remarkable ability of acquiring further mechanisms of resistance to multiple groups of antimicrobial agents. *P. aeruginosa* represents a phenomenon of antibiotic resistance, and demonstrates practically all known enzymic and mutational mechanisms of bacterial resistance[4]. Often these mechanisms exist simultaneously, thus conferring combined resistance to many strains [5].

P. aeruginosa is intrinsically resistant to many structurally unrelated antimicrobial agents [6], because of the low permeability of its outer membrane (1/100 of the permeability of E. coli outer membrane)[7], the constitutive expression of various efflux pumps with wide substrate specificity [8] and the naturally occurring

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chromosomal AmpC β-lactamase [9]. The natural resistance of the species relates to the following β -lactams: penicillin G; aminopenicillins, including those combined with β-lactamase inhibitors; first and second generation cephalosporins. *P. aeruginosa* easily acquires additional resistance mechanisms, which leads to serious therapeutic problems.

As carbapenems are among the most reliable therapeutic options for treating *Pseudomonas aeruginosa* infection, the finding of carbapenem resistance is an ominous development that challenges this "last resort antibiotic".

Production of metallo-β-lactamases (MBLs) is an important mechanism of carbapenem resistance, not only because MBLs can hydrolyse most β-lactams including carbapenems, but MBL-producing *P. aeruginosa* are frequently also multidrug-resistant (resistant to ≥3 classes) [10]. Usually, most MBL-producing *P. aeruginosa* strains are resistant to other antibiotic classes, including fluoroquinolones and aminoglycosides, often leaving polymyxins as the sole therapeutic options [11].

That is why early detection of carbapenemase producers is important. This is not always easy, taking into account that those resistance determinants may sometimes confer only a slight increase of MIC values for carbapenems, and this is the reason why using molecular approaches and not only phenotypic tests are sometimes very helpful [12].

We have performed this study to find out the frequency of MBL- producing *Pseudomonas aeruginosa* in our area and their antibiotic profile.

METHODOLOGY

1. Bacterial isolates:

A total of 120 specimens non- repetitively and consecutively obtained from clinical specimens in Suez Canal University Hospital from July 2015 to December 2015. *P. aeruginosa* were isolated and identified conventionally by culturing on citrimide agar (Himedia, India) and using biochemical tests. The isolates were stored at -20°C in trypticase soy broth containing 20% glycerol.

2. Antimicrobial susceptibility testing:

Antimicrobial susceptibility tests of isolates to different antibiotics were performed using disc diffusion method recommended by the clinical and Laboratory Standards Institute (CLSI) guidelines [13] as shown in table 1. All antibiotic used in were purchased from sigma.

Table 1: antibiotics used for *Pseudomonas aeruginosa* by Kirby-Bauer method

Antimicrobial agent	Disk content	S	I	R
Piperacillin	100 πg	≥21	15-20	≤14
Ceftazidime	30πg	≥18	15-17	≤14
Cefepime	30πg	≥18	15-17	≤14
Aztreonam	30πg	≥22	16-21	≤15
ciprofloxain	5 πg	≥21	16-20	≤15
Meropenem	10 πg	≥19	16-18	≤15
imipenem	10 πg	≥19	16-18	≤15
Gentamicin	120πg	≥15	13-14	≤12
Colistin	10 πg	≥11	-	≤10
Amikacin	30πg	≥17	15-16	≤14
Levofloxacin	5 πg	≥17	14-16	≤13

3. Phenotypic detection of ESBL:

a. Screening tests:

Muller Hinton agar plate was inoculated with a 0.5 McFarland suspension of fresh overnight blood agar culture of the test strains. Cefotaxime (30μg) and ceftazidime (30μg) disks were placed on the agar surface and incubated for 24 hours at 37°C. If the inhibition zone diameter was ≤22mm for ceftazidime and ≤27mm for cefotaxime, the strain was considered as potential producer of ESBLs [13]

b. Confirmatory test:

- Cefotaxime and ceftazidime (30μg) were added alone in one half of the Muller Hinton plate, and in combination to clavulanic acid (10μg) on the other half of the plate. Isolates that showed increase in the diameter of the zone of inhibition in the presence of clavulanic acid by ≥5mm than that without clavulanic acid were considered as ESBLs producer strains [13].
- Double Disk Synergy Test (DDST): ceftriaxone or cefotaxime (30μg) were placed at distances (20 mm center to center) around amoxicillin-clavulanic acid (20/10μg) disk. Enhancement of inhibition zone towards the amoxicillin-clavulanic acid disk indicating a synergy between clavulanic acid and any one of the tested antibiotics was taken to confirm ESBL production [13].

4. Phenotypic detection of metalo-beta lactamases (MBL):

MBL was suspected when the isolate was resistant to meropenem and imipenem [13].

Methods used for screening of MBL:

a. Screening test:

The isolates that showed an inhibition zone diameter of 16–21 mm around meropenem antibiotic disk (10µg) and/or 19–21 mm around imipenem disk (10µg) were considered as potential producer of carbapenemase [13].

b. Confirmatory test (Modified Hodge test):

The meropenem resistant strains were subjected to Modified Hodge test (MHT) for detection of carbapenemases . An overnight culture suspension of *E. coli* ATCC 25922 adjusted to 0.5 McFarland, diluted 1:10 in saline was spread on MH agar plate (15 cm) and allowed to dry. Meropenem disk (10µg) was placed at the center of the plate. Three to five colonies of *P.aeruginosa* test organisms grown over-night on blood agar plate were streaked in a straight line from the edge of the disk to the periphery of the plate in four different directions. The plate was incubated at 37°C. Both positive and negative control strains were inoculated in the same way on each plate. The presence of a cloverleaf shaped zone of inhibition due to carbapenemase production was considered as positive[14]. The isolates that show positive Modified Hodge test were then tested for the presence of class B carbapenemases by using the EDTA disk synergy test.

c. EDTA disk synergy test:

EDTA solution (0.5 M) was prepared by dissolving 186.1 of sodium EDTA.2H2O in 1 L of distilled water and the PH was adjusted to 8 by using NaOH then sterilized by autoclave. Two meropenem antibiotic disks (10µg) were placed on Muller Hinton agar surface, to one of them, 10µl of the prepared EDTA solution were added, while the other was left as it is. An increase in the diameter of the zone of inhibition around the meropenem-plus-EDTA disk by ≥5mm, more than the meropenem disk alone indicated the presence of class B carbapenemases (MBL-producer) [15].

5. Genotypic analysis of MBL determinants:

Polymerase Chain Reaction (PCR) testing for all isolates for MBL genes (bla_{IMP} and bla_{VIM}) was done.

a. DNA extraction

DNA extraction was done from overnight bacterial growth solution by using GeneJET genomic DNA purification kit (Fermentas) according to manufacturer instructions. The concentration of DNA was measured by spectrophotometer.

b. Detection of MBL genes:

The PCRs were carried out in volumes of 20 µl containing 1 µM of each primer (10 pmol) as shown in Table (1), 10µl master mix, and 2 µl of the extracted DNA, made up to a total volume of 20 µl with pure, sterile double-distilled water.

The PCR cycling conditions were as follows [16]:

- for *bla* $_{IMP}$: 94 °C for 10 min followed by 35 cycles of 94 °C for 30 sec, 50 °C for 45 sec, and 72 °C for 1 min followed by final extension of 72 °C for 10 min.
- For *bla* $_{VIM}$: 94°C for 10 min followed by 35 cycles of 94 °C for 30 sec, 55 °C for 45 sec, and 72 °C for 1 min followed by final extension of 72 °C for 10 min.

The amplifications were carried out in a Gradient Thermal Cycler (MyCycler, BIO-RAD).

c. Detection of the amplified products:

The resulting products were separated in 1 % agarose gels and 1× Tris-acetate-EDTA buffer and stained with ethidium bromide, and then images were captured using the Syngene G: Box documentation system. The amplified products were identified by comparing the band size to the expected sizes as shown in table 1.

Table 2: The primers used in the study (Pitout et al., 2005)

Primers*	Sequence (5` to 3`)	Products size	Target Gene
IMP F	5`-GAAGGCGTTTATGTTCATAC - 3`	587 bp	bla_{IMP}
IMP R	5`-GTATGTTTCAAGAGTGATGC- 3`		
VIM F	5`- GTTTGGTCGCATATCGCAAC-3`	382 bp	bla_{VIM}
VIM R	5`- AATGCGCAGCACCAGGATAG-3`		

RESULTS

A total of 120 non duplicate isolates were collected from different wards in our hospital. Out of these isolates, 26 were diagnosed as *P. aeruginosa*.

Antimicrobial susceptibility testing:

All of the 26 isolates (100%) were resistant to imipenem and meropenem , while resistance was 42.3% for cefepime, and 88.5% for ceftazidime.

Table 3: antibiotics used for *Pseudomonas aeruginosa* by Kirby-Bauer method

Antimicrobial agent	Disk content	S	I	R
Piperacillin	100 πg			
Ceftazidime	30πg	3 (11.5%)	0	23 (88.5%)
Cefotaxime	30πg	0	0	26 (100%)
Cefepime	30πg	11 (42.3%)	4 (15.4%)	11 (42.3%)
Aztreonam	30πg	21 (80.8%)	2 (7.7%)	3 (11.5%)
ciprofloxain	5 πg	17 (65.4%)	1 (3.9%)	8 (30.7%)
Meropenem	10 πg	0	0	26 (100%)
imipenem	10 πg	0	0	26 (100%)
Gentamicin	120πg	22 (84.6 %)	0	4 (15.4%)
Ceftriaxone	30πg	6 (23%)	0	20 (77%)
Colistin	10 πg	23 (88.5%)	0	3 (11.5%)
Amikacin	30πg	22 (84.6 %)	0	4 (15.4%)
Levofloxacin	5 πg	21 (80.8%)	0	5 (19.2%)

Phenotypic detection of ESBL:

According to CLSI criteria, out of 26 *P.aeruginosa* isolates, only 3 (11.5%) isolates (11.54%) were sensitive to ceftazidime (inhibition zone diameter >22 mm) and none of them were sensitive to cefotaxime (inhibition zone diameter for all isolates were ≤27mm). By using confirmatory tests, we found that 80.8% (21 strains out of 26 strains) were ESBL-producers.

Phenotypic detection of metalo-beta lactamases:

All isolates (100%) were considered as potential producer of carbapenemase as the inhibition zone diameters around meropenem antibiotic disk were 16–21 mm and/or 19–21 mm around imipenem disk. By MHT and EDTA synergy test, only 53.8% (14 out of 26) were considered as class B carbapenemase (MBL-producing).

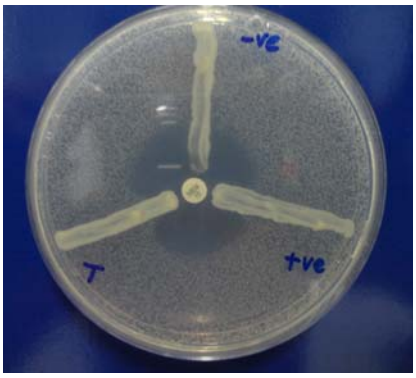

Fig. 1: Modified Hodge test

Detection of MBL genes:

All isolates confirmed to be MBLs producers by phenotypic methods (14 strains) were subjected to PCR using two sets of primers; a primer for the bla_{IMP} gene and another for the bla_{VIM} gene.

As shown in Figure (2), six isolates (23% of pseudomonas isolates) were positive for MBL production using the bla_{IMP} PCR assay; positive specimens showed specific bands of approximately 587 bp in size. While, seven isolates (27% of pseudomonas isolates) were positive for MBL production using the bla_{VIM} PCR assay (Figure 3); with specific bands of approximately 382 bp in size.

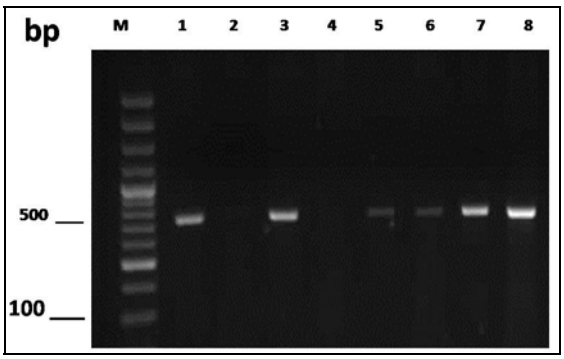

Fig. 2: Results of bla IMP PCR assays:
Agarose electrophoresis showed PCR products of the bla IMP of some tested isolates. Lanes: M, molecular weight marker (Axygen Biosciences); All lanes (except lane 2 and 4) are positive strains for this gene. Positive specimens showed specific bands of approximately 587 bp in size

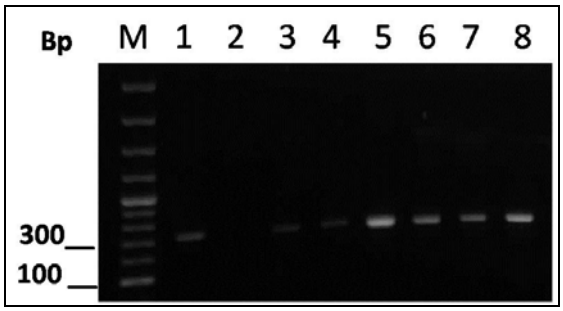

Fig. 3: Results of bla VIM PCR assays:
Agarose electrophoresis showed PCR products of the bla IMP of some tested isolates. Lanes: M, molecular weight marker (Axygen Biosciences); All lanes (except lane 2) are positive strains for this gene. Positive specimens showed specific bands of approximately 382 bp in size

The relation between MBL genes and resistance pattern of the isolates to cephalosprines, Aztreonam and gentamicin is shown in table (4). We noticed that all IMP/VIM positive strains (4 strains) were resistant to ceftazidime, cefotaxime, gentamicin and ceftriaxone. Three of them were resistant to Aztreonam with no statistical significance difference (p value = 0.9).

Table 4: Distribution of isolates resistant to third generation cephalosporins, aztreonam and gentamicin and its relation to MBL state.

MBL state (No.)	*Antimicrobial agents (No. of resistant isolates) **				
	Ceftazidime (32)	**Cefotaxime (26)**	**Ceftriaxone (20)**	**Gentamicine (4)**	**Aztreonam (3)**
IMP positive (6)	6	6	6	4	3
VIM positive (7)	4	7	5	4	3
IMP/VIM positive (4)	4	4	4	4	3
VIM positive/ IMP negative (3)	1	3	2	0	0
IMP positive/ VIM negative (2)	2	2	2	0	0

*** P value = 0.9**

DISCUSSION

Pseudomonas aeruginosa infections are commonly associated with high patient morbidity and mortality rates. Metallo β-lactamase (MBL) producing Pseudomonas raise special concerns as these isolates are responsible for several nosocomial outbreaks and high rate of clinical failure among infected patients. The 1st MBL producing Pseudomonas was reported from Japan in 1991 & since then it has been described from various parts of the world, including Asia, Europe, Australia, South America & North America [17, 18].

Different studies around the world show that there is a wide variation in the prevalence of MBLs.

In the present study, we found that the prevalence of MBL producers in Pseudomonas is around 53.8%. This high rate was in accordance with the results of Lucena et al. who reported a prevalence of 69% for MBL [19] and those of Lagatolla and colleagues who found 70% MBL in *P. aeruginosa* [20].

On the other hand, many studies showed lower rates of MBL production, such as those conducted by Deshpande et al (10-30%) [21], Variaya et al (20.8%) [22], Navneeth et al (12%) [23], Rajput et al (12%) [24].

In our study, we observed that our isolates of *Pseudomonas aeruginosa* showed high rates of resistance to various antibiotics such as ceftazidime (88.5%), cefepime (42.3%) ceftriaxone (77%), cefotaxime (100%). Our findings were more or less similar to those observed by NagKumar et al [25], where they reported an increased resistance of this organism to various antibiotics like Ceftazidime (46.9%) and Cefepime (49%), Gentamicin (52.5%) & Ciprofloxacin (46.4%). Also, Peshattiwar et al reported 50.79% resistance of Cephotaxime [26] and Dwivedi et al, who reported a 63% resistance to Ceftazidime [27].

Production of multiple β-lactamases by *P. aeruginosa* has tremendous therapeutic consequences and poses a significant clinical challenge if it remains undetected. Since these organisms also carry other drug-resistance genes, early identification of those infections is necessary as the appropriate treatment might reduce the spread of these resistant strains as well as reduce the mortality in hospitalized patients. This emphasizes the need for the detection of isolates that produce these enzymes to avoid therapeutic failures and nosocomial outbreaks.

MBLs represent a clinical threat due to their broad spectrum of activity and their resistance to beta-lactamase inhibitors. MBLs are associated with increasing rates of clinical failure among infected patients. Therefore, all isolates of *P. aeruginosa* resistant to imipenem or meropenem should be screened for MBL production.

Lack of awareness of the problem of the alarming rise in the multiresistance. This leads to urgent requirement of strict strategies for intervention to limit inappropriate uses of antibiotics and to improve infection control measures to prevent an increase in these nosocomial infections.

In conclusion, bla IMP and blaVIM MBLs were isolated in high rates (23% and 27% respectively) from *pseudomonas aeruginosa* isolated from cases of nosocomial infection in Suez Canal University, Ismailia, Egypt. Moreover, this study provides a new report for detection MBLs genes in Suez Canal University Hospital and also the findings in the present study will provide a guide for proper selection of antibiotics and also to provide strict infection control measures to combat nosocomial infections.

Conflict of Interest:

The authors declare that they have no conflict of interest.

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