Original Article

High incidence of MBL-mediated imipenem resistance among *Pseudomonas aeruginosa* from surgical site infections in Egypt

Marian Refat Raouf[1], Mohamed Sayed[1], Hazim Ahmed Rizk[1], Noha Anwar Hassuna[1]

[1] Medical Microbiology and Immunology Department, Faculty of Medicine, Minia University, Minia, Egypt

Abstract

Introduction: Surgical wound infection is a serious problem, especially with metallo-beta lactamases (MBLs)- producing gram-negative bacteria as *Pseudomonas aeruginosa*. The main objective of this work was to evaluate for the first time in Minia- Upper Egypt, the incidence of imipenem-resistant *Pseudomonas aeruginosa* infection of surgical wounds particularly that mediated by MBL production.

Methodology: *P. aeruginosa* was isolated from infected wounds by swabs and underwent full microbiological identification and Antibiotic Susceptibility testing. MBL production was tested by E-test and PCR was used for imipenemase *(bla*$_{IMP}$*)* and Verona integron-encoded metallo-beta-lactamase (*bla*$_{VIM}$) gene detection.

Results: Out of 200 pus samples collected from surgical site infections, *P. aeruginosa* had the prevalence rate of 35%. Imipenem resistance was found in 28.57% of the isolated *Pseudomonas aeruginosa*. The prevalence of MBL-producing isolates among Imipenem-resistant *P. aeruginosa* (IRPA) was 85 % by phenotypic method with 29% of them harboring *bla*$_{VIM}$ gene. High resistance rates to other classes of antibiotics were reported among the isolates with multi-drug resistance (MDR) detected in 97.3% of the isolates.

Conclusion: To our knowledge, this is the first report in Minia, Upper Egypt describing the relatively high incidence of IRPA in infected surgical wounds with MBLs involved in the majority of isolates.

Key words: *Pseudomonas aeruginosa;* imipenem resistance; metallo-beta lactamase.

Introduction

Surgical site infection (SSI) is the most common and most challenging healthcare-acquired infection worldwide. Various organisms are responsible for SSI with *Staphylococcus aureus* and *Pseudomonas aeruginosa* being the most commonly isolated species [1,2].

P. aeruginosa is an opportunistic pathogen, which is associated with high incidence of morbidity and mortality due to the high resistance to a wide array of antimicrobials [3]. The mechanisms of resistance are various and are usually contemporaneous [4] imposing a great challenge for choosing an appropriate antimicrobial. The most suitable antimicrobials used against multidrug resistant (MDR) *P. aeruginosa* are the carbapenems, which has capability to resist extended-spectrum β-lactamases (ESBLs) [5]. However, the upsurge of metallo-beta-lactamases (MBLs) producing *P. aeruginosa* has led to developing resistance to all β-lactams including carbapenems, posing a great limit in choosing the right regimen and a great burden requiring strict infection control measures [6]. MBLs are β- lactamases that possess catalytic Zn^{2+} ions unlike the serine residue in serine β- lactamases [7], hence they are inhibited by zinc ion chelators, such as EDTA. The MBLs display a wide range of activity against all the β-Lactams except monobactams, e.g. aztreonam. MBL genes in *P.aeruginosa*, are usually carried with other antimicrobials resistance genes on integrons, leading to the development of multi-drug resistance [8]. The commonly reported MBLs are: imipenemase (IMP), Verona integron-encoded metallo-beta-lactamase (VIM), and New Delhi metallo-beta-lactamase (NDM) [9].

The aim of this study was to assess the presence of metallo-β-lactamase-producing *P. aeruginosa* (MPPA) among SSI isolates from various surgery wards and to evaluate the presence of *bla*$_{VIM}$ and *bla*$_{IMP}$ genes in Upper Egypt.

Methodology

This cross-sectional study was done in the Microbiology and Immunology Department, Faculty of Medicine. A cluster sample by time was collected including pus samples from postoperative (2-15 days) patients hospitalized during the period between May

and December 2015 in various surgical departments including General Surgery, Urology, Orthopedics and Burn Units of three different hospitals in Minia including Minia University Hospital (tertiary care referral teaching hospital), Minia General Hospital and Minia Health Insurance Hospital. These hospitals constitute the main hospitals in Minia governorate that have surgical departments. According to the risk of contamination, surgical wounds were classified by National Academy of Sciences into clean, contaminated, clean contaminated and dirty [10]. Pus samples were collected from patients whose postoperative wound showed pus with inflammatory signs as redness, warmth and edema. A written informed consent was obtained from patients before inclusion in the study. The surgical wound was open in all the cases and was only closed when complete resolution of infection occurs. A sterile closed dressing was used and was changed three times daily till healing.

The study was ethically approved by the faculty of Medicine and was carried out according to the principles expressed in the Declaration of Helsinki.

Sample collection
Swab samples were placed in sterile Aime's transport medium (Thermo Fisher, Hampshire, UK) and were put in an ice pack box and transported immediately to the laboratory within 2 hours.

Isolation and identification
P. aeruginosa was isolated by culturing the swab samples on cetrimide agar (Biolife, Milan, Italy). The isolates were then further confirmed by microscopic examination and biochemical reactions: oxidase, citrate, Voges–Proskauer and triple sugar iron tests.

Antibiotic susceptibility
Susceptibility to various antimicrobials was tested by Kirby-Bauer disk diffusion method on Muller-Hinton medium using the following antibiotic discs: cefepime (FEP) 30 µg, ceftazidime (CAZ) 30 µg, imipenem (IPM) 10 µg, amikacin (AK) 30 µg,

gentamicin (CN) 10 µg, ciprofloxacin (CIP) 5 µg, ampicillin 10 µg (AM), Sulphamethoxazole-trimethoprime (SXT) 25 µg, chloramphenicol (CAM) 30 µg (Oxoide, Hampshire, England).

E test for MBLs production
The E-test MBL strips (Liofilchem, Roseto degli Abruzzi, Italy) have seven dilution ranges of IPM (4 to 256 µg/mL) and IPM (1 to 64 µg/mL) with a constant concentration of EDTA. Minimal Inhibitory Concentration (MIC) end points were read at the site of strip intersection by the inhibition ellipses.

An MIC ratio of imipenem/imipenem + EDTA ≥ 8 was considered positive result. In addition, the presence of a phantom zone or a deformation of the imipenem ellipse was also considered a positive result [11].

DNA extraction and PCR
Genomic DNA was extracted using Gene JET Genomic DNA extraction kits (Thermo scientific, Vilnius, Lithuania). Amplification was done for blaIMP-1, blaIMP-2, blaVIM-1 and blaVIM-2 genes using primers in Table 1. PCR was carried out in 25 µL reactions using 12.5 µL of PCR master mix (Nippon genetics, Europe), 1 µL (12.5 pmol) of each primer, 2µL (20ng) of template DNA and 8.5 µL of sterile water. The amplification reactions were carried out by conventional PCR using Biometra, UNO II thermal cycler under the following conditions: for blaIMP-1, blaIMP-2 and blaVIM-1; initial denaturation at 94°C for 5 minutes, followed by 35 cycles each consists of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72 °C for 90 seconds with a final extension step at 72°C for 10 minutes; for blaVIM-2 gene the same conditions were used except that the initial denaturation was for 3 minutes [12,13]. The resulting products were loaded in 1.5% agarose gel and were visualized under UV illumination system.

Statistical analysis
Statistical analysis was performed by the Statistical Package for Social Science (SPSS) version 16.0 (IBM,

Table 1. Primers for PCR detection of MBL genes.

Gene	Primer	Sequence	Amplicon (bp)	Reference
bla_{IMP-1}	Forward 5'-3'	CTA CCG CAG CAG AGT CTT TG	587	[14]
	Reverse 5'-3'	AAC CAG TTT TGC CTT ACC AT		
$bla_{IMP 2}$	Forward 5'-3'	GTT TTA TGT GTA TGC TTC C	678	[15]
	Reverse 5'-3'	AGC CTG TTC CCA TGT AC		
bla_{VIM-1}	Forward 5'-3'	AGT GGT GAG TAT CCG ACA G	261	[16]
	Reverse 5'-3'	ATG AAA GTG CGT GGA GAC		
$bla_{VIM 2}$	Forward 5'-3'	ATG TTC AAA CTT TTG AGT AAG	801	[17]
	Reverse 5'-3'	CTA CTC AAC GAC TGA GCG		

USA). Graphics were performed using Excel 2007 (Microsoft Inc., USA). For descriptive statistics, data were presented as frequency and percentage. For analytical statistics, student's t test was used to compare quantitative variables. Levels of significance determined by *p* value were interpreted as follows: $p \leq 0.01$ and ≤ 0.05 indicating high significance and significance, respectively.

Results

Full bacteriological identification was done for all samples for detection of *P. aeruginosa*. Positive isolates were subjected to antibiotic susceptibility testing with different drugs. Isolates that showed resistance to imipenem antibiotic were tested for MBLs enzyme production phenotypically by E test and genotypically by PCR. All results were statistically analyzed.

Out of 200 samples from infected surgical wounds, 70 *P. aeruginosa* isolates were identified (35%). According to the type of wound and operation: 26 of the isolates were obtained from abdominal incisions (37.1%), 7 from hernioplasty incisions (10%), 15 from traumatic wound repairs (21.4%), 4 from post-mastectomy wounds (5.7%) and 18 from amputation sites (25.7%). Only 4 of the isolates were obtained from wounds of the clean type (5.7%), 7 were from clean contaminated wounds (10%), while the majority (59 isolates: (84.3%)) was from contaminated wounds (Table 2). The average length of hospital stay was 7 days for positive cases compared to 5 days for negative cases and the difference was statistically significant (p < 0.0001). No risk factors such as diabetes mellitus or malignancy were reported in 72.9% patients, while 22.9% had diabetes mellitus and only 4.3% had malignancy.

According to disc diffusion method, a high resistance rate was detected against cefepime, ceftazidime, ampicillin (all the isolates), chloramphenicol, Sulphamethoxazole-trimethoprim (68 isolates), gentamycin (62 isolates), amikacin (58 isolates) and ciprofloxacin (51 isolates), while a much lower resistance rate was observed against imipenem (20 isolates) (Figure1).

Figure 1. Antibiotic resistance profile of *P. aeruginosa* isolates.
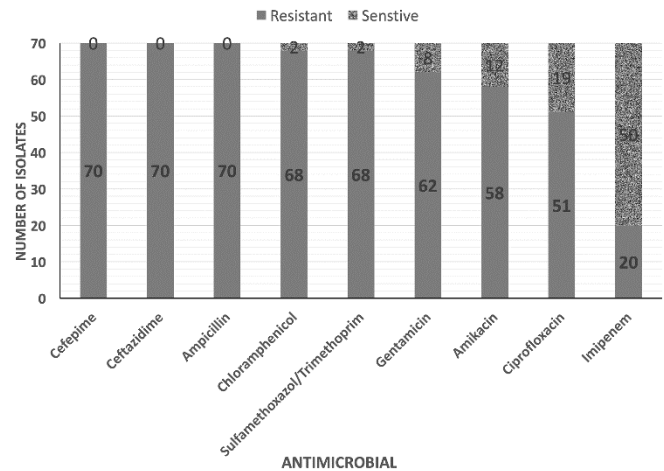
The imipenem resistant *P. aeruginosa* isolates (20 isolates) were isolated with the following frequencies from various wound types: 8 isolates were from infected abdominal incisions (40%), 6 were from amputation sites (30%), 3 were from trauma repair (15%), 2 were from mastectomy wounds (10%) and 1 was from a hernioplasty wound.

Regarding phenotypic detection of MBLs, among the 20 imipenem resistant isolates, 17 isolates (85%) showed positive result in E-test, while 3 turned out to be negative (15%).

As for the detected MBL genes, *bla*$_{VIM -1}$ gene was detected in 29% of MBL positives isolates, while *bla*$_{VIM-2}$ gene was detected in 5%. On the other hands, *bla*$_{IMP}$ genes were not detected in any of the isolates (Table 3).

Discussion

The escalation in the spread of MBLs among members of non-fermenting gram-negative bacteria, particularly *P. aeruginosa* poses a therapeutic challenge. These enzymes can hydrolyze all β-lactams including carbapenems; the last resort of antibiotics used for the management of severe infections caused by ESBL producing bacteria [18].

In this study, *P. aeruginosa* was detected in 35% of 200 swab samples from SSI from different surgical

Table 2. Culture results according to the type of surgical wound.

	Positive		Negative		Total		*P* value
	No	**%**	**No**	**%**	**No**	**%**	
Clean-contaminated	7	15	40	85	47	100	
Clean	4	12	30	88	34	100	0.0001
Contaminated	59	49.5	60	50.5	119	100	
Total	**70**		**130**		**200**		

Table 3. The presence of bla_{VIM} gene in IRPA isolates in relation to MBL E-test results.

| E-test | VIM genes | | | | Total |
| | bla_{VIM-1} | | bla_{VIM-2} | | |
	Negative	Positive	Negative	Positive	
Positive	12	5	16	1	17
Negative	0	3	3	0	3
Total	**12**	**8**	**19**	**1**	**20**

wards in the three hospitals. Another study has found a similar infection rate of *P. aeruginosa* done in a different region of Egypt [19]. The relatively high incidence of wound infection could be due to either intrinsic organisms especially in wounds of contaminated nature or could be due to intraoperative or postoperative infection. A high incidence of MDR was detected in 97% of the isolates. The increase in MDR has been observed in many studies in Egypt [20,21]. This high level of resistance could be explained by the lack of proper infection control measures and the extensive abuse of different antimicrobials. For instance, a study carried out in Minia showed that β-lactams and macrolides were commonly prescribed both by doctors and pharmacists for common cold [22]. As a matter of fact, antibiotics could be easily purchased without doctor's prescription in Egypt (what is known as patient's self-medication) [23].

In this study, imipenem (IPM) resistance, as tested by disc diffusion method, was detected in 20 isolates out of 70 *P. aeruginosa* isolates (28.57%). This result is similar to that of Ali and Abdel-Razik, where imipenem resistance was detected in 28.6 % of their *P. aeruginosa* isolates [24]. Although relatively high, this incidence of imipenem resistance was lower than that reported by other studies carried in Egypt (72%) [25] and in other countries like India (40% in one study [26] and 57.8% in another study [27]) and Brazil (34.5%) [28].

Owing to several factors, such as stability against extended-spectrum β-lactamases (ESBLs), high affinity to penicillin-binding proteins, carbapenems have been described as the most potent β-lactams against MDR *P. aeruginosa*. The susceptibility to carbapenems in *P. aeruginosa* varies considerably according to the geographic distribution *P. aeruginosa* [6]. Various mechanisms could be responsible for carbapenems resistance in *P. aeruginosa* including the decrease in the permeability of the cell-wall, intrinsic efflux systems over-expression in addition to production of hydrolyzing enzymes particularly MBLs. The presence of MBLs is responsible for about 40% of IRPA isolates all over the world; 39% in a surveillance program in Italy [29] and 40% in Portugal [30], with higher rates

reported in Pakistan (764.9%) [31] and remarkably lower rates in France (5.6%) [32].

In addition to their resistance to the commercially available β -lactamase inhibitors like clavulanate, tazobactam, and sulbactam [33], the great challenge with MBLs is the lack of a clinical inhibitor up-to-date due to the heterogeneity of MBLs, and the similarities of the active site fold with mammalian enzyme (ex; human glyoxalase-II) [34].

A remarkably higher prevalence of MBL is detected in this study with 85% of the IRPA producing MBLs, which is comparable to few studies done in Egypt and India [25,26,35]. This could be attributed to the regional difference in MBL distribution among IRPA worldwide [36].

Genes encoding MBLs are mostly acquired by horizontal gene transfer carried by plasmids or transposons leading to a wider prevalence of MBLs as a cause of carbapenems resistance [37,38]. The first detected MBLs were the IMP- and VIM-type enzymes, followed by more types of acquired MBLs, including the SIM-, NDM-, SPM-, KHM-, GIM-, AIM-, DIM-, SMB-, TMB-, and FIM-type enzymes. Among them, IMP-, VIM-, SPM-, GIM-, NDM- and FIM-type variants have been continuously reported in *P. aeruginosa* [39]. bla_{VIM} genes were detected in this study, while none of the isolates were bla_{IMP} producers judged by PCR using specific primers for bla_{IMP} genes. The presence of bla_{VIM-2} was reported in previous studies in Egypt, Europe, and East Asia [25,40]. Diab and colleagues, similarly, detected no bla_{IMP} genes among MBLs-producing isolates [25], however; other studies found bla_{IMP} but to a lesser extent than the bla_{VIM} genes [41,42]. Interestingly, bla_{VIM-1} was detected in three isolates, which were negative for MBL production (Table 3), this could be due to the possibility of missing borderline cases by MBL E-test as reported by Walsh and colleagues [11]. The lack of bla_{VIM} and bla_{IMP} genes in MBL isolates should be further investigated for other genes encoding MBL production [38]. It is also worth noting here to mention that the presence of bla_{VIM} genes is not per se enough to conclude that MBL production is attributed to them as

further sequencing would be required to confirm the absence of mutations affecting the gene functionality.

The New Delhi metallo-beta-lactamase gene (*bla*NDM) is quite rare in Egypt with only a case study reporting the occurrence of this gene in Egypt prior to the start of our study [43], hence we did not look for this gene in the current study. However, we could look for it in further studies.

Conclusion

In conclusion, this study sheds the light on the upsurge of IRPA isolated from surgical wounds and demonstrates that MBL production is a major mechanism among them. This mandates proper implementation of infection control measures and appropriate use of antimicrobials. Further investigations should been invoked to determine the clonality of the strains found in this study and to evaluate other imipenem-resistance mechanisms.

Authors' Contributions

NAH, MR, MS and HR have devised and designed the study. NAH and MR carried out the experimental work and gathered the data. NAH, MR, MS, and HR made data analysis and interpretation. NAH and MR wrote the manuscript. NAH, MS and HR critically revised the manuscript.

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Corresponding author
Noha A Hassuna, MD, PhD, Associate Professor,
Dept. of Microbiology and Immunology, Faculty of Medicine,
Minia University,
Minia 61511, Egypt.
Phone: 00201274141444
Email: nohaanwar@mu.edu.eg

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