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Phenotypic detection of Metallo-β-Lactamase (MBL) in Imipenem-Resistant *Pseudomonas aeruginosa*, a study from a tertiary care hospital in Peshawar, Pakistan

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Abstract

Production of metallo-β-Lactamase (MBL) by *Pseudomonas aeruginosa* has emerged as one of the most clinically worrisome resistance mechanisms. The present study aimed phenotypic detection of MBL production in clinical isolates of Imipenem resistant *P. aeruginosa*. About 245 non-duplicated *P. aeruginosa* isolates were collected during a ten-month study duration from a tertiary care hospital of Peshawar, Pakistan. The isolation of the *P. aeruginosa* isolates was done from high vaginal swab, pus, urine, sputum, blood, and other body fluids. Antibiotic susceptibility profile of the tested isolates was investigated by Kirby-Bauer disc diffusion method. The isolates were further screened for MBL production by Imipenem- EDTA combined disc test (CDT). Among 245 clinical isolates, percentage of outdoor and admitted patients were 62% and 37%, whereas gender-wise ratio includes 57% male and 42% female patients respectively. Burn unit showed highest number of *P. aeruginosa* isolates among admitted patients, followed by endocrinology, surgical, orthopedics, gynecology, urology, ENT, skin, pediatrics, and medical ward. Out of total isolates, 15% isolates showed resistance towards Imipenem by Kirby-Bauer method. Thirty-two (13%) out of 245 isolates were found positive for MBL production by CDT method. All MBL producers showed a notable resistance against imipenem, cefepime, aztreonam while sensitivity was observed towards all the other tested antibiotics. The *P. aeruginosa* isolates are rapidly developing resistance against effective therapeutic agents especially carbapenem, which is of serious concern. Therefore, rapid detection of MBLs in *P. aeruginosa* isolates is essential for controlling the spread of MBL-encoded genes and efficient treatment of patients.

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Introduction

Pseudomonas is a group of Gram-negative, motile, non-spore producing, and aerobic bacilli consisting of more than 140 species, majority of which are saprophytes. A number of Pseudomonas species causing infections in humans includes *Pseudomonas aeruginosa*, *P. putrefaciens, P. Putida, P. Maltophilia, P fluorescens*, and many others (Ryan and Ray, 2004). *Pseudomonas aeruginosa* comprises 80 percent of Pseudomonas recovered from clinical specimens (Pfaller and Herwaldt, 1997). *Pseudomonas aeruginosa* causes hospital associated infections (HAIs) and is a major threat to hospitalized patients mainly, patients having serious underline problems such as cancer or burns (Baltch and Smith, 1994).

According to National Nosocomial Infections Surveillance System, *P. aeruginosa* is ranked as third most common pathogen that accounts for almost 10.1% of all nosocomial infections (Hidron *et al.*, 2008). *P. aeruginosa* have the ability to evolve new strategies to invade its compromised host. Also, mutations resulting resistance to one antibiotic can lead to ineffectiveness of whole class of drugs (Giamarellou and Poulakou, 2009; Lagatolla *et al.*, 2004).

Carbapenems, members of beta-lactam class of antibiotics includes Imipenem, meropenem and ertapenem, used as a first drug of choice for treating infections caused by multi-drug resistant (MDR) Gram-negative bacteria, specially, *P. aeruginosa* (Birnbaum *et al.*, 1985; Breilh *et al.*, 2013). During recent decades, resistance against carbapenems has been increased drastically and reported from all over the world (Kazmierczak *et al.*, 2016). Further Studies suggested that this type of resistance occur due to evolution of "carbapenem-hydrolyzing enzymes" in many Gram-negative bacilli including *Pseudomonas aeruginosa* (Rasmussen and Bush, 1997).

Various beta lactamases have been identified so far. Among them, the genetically motile metallo-β-Lactamases (MBLs) are the most versatile ones because they have the ability to hydrolyze all beta lactams (Patzer *et al.*, 2009). Metallo-β-Lactamases that require zinc or other heavy metals for their catalytic activity are named metallo-β-Lactamases. The metabolic activity of these enzymes is inhibited by the addition of metal chelating agents such as EDTA (Ethylene diamine tetra acetic acid) and thiol-based compounds (Pitout *et al.*, 2007).

Genes that code for MBL production is thought to be carried by large mobile plasmids or transposons, provide ability to transfer MBL genes to other bacterial species through horizontal gene-transfer (Pitout *et al.*, 2007). Until now five different types of acquired MBL genes have been identified. These include IMP, VIM, SPM, GIM, and SIM type genes. Among them SPM, GIM, and SIM type genes are restricted to certain geographical areas, while IMP and VIM variants have been reported across the globe (Queenan and Bush, 2007).

Polymerase chain reaction (PCR) remains gold stranded for the detection of MBL production in clinical isolates (Franklin *et al.*, 2006), but they are seldom used in diagnostic laboratories mainly in tropical and developing countries like Pakistan, and if used, they are restricted to academic and research purpose only. Most of these laboratory's relay on Culture based phenotypic methods for MBL's detection.

Early detection of the MBL producing microorganisms is necessary to ensure appropriate antibiotic use and to observe infection control measures. It is well evident that activity of MBLs is dependent on zinc or cadmium. Many screening methods involve metal chelating agents, like that of ethylene diamine tetra acetic acid (EDTA) and thiol-based compounds for example; 2-mercaptopropionic acid (2- MPA), which have the ability of restricting MBL activity, and have been developed to detect MBL-producing organisms (Sharma *et al.*, 2015).

The present study aimed to detect susceptibility profile of MDR *Pseudomonas aeruginosa* and its

MBL-producing ability through Imipenem-EDTA combine disc test (CDT) method.

Materials and methods

Collection and Identification of P. aeruginosa isolates

The present study was conducted in the Microbiology Laboratory of Lady Reading Hospital Peshawar, Pakistan, during August 2015 to May 2016. Specimens (high vaginal swab, pus, urine, sputum, blood, and other body fluids) were obtained from the indoor and outdoor patients of different clinical units, include, burn unit, endocrinology, surgical, orthopedics, gynecology, urology, ENT, skin, pediatrics, and medical ward. For inoculation and isolation purpose, routine culture medias such as Cystine electrolyte deficient media (CLED), MacConkey agar, Blood agar, and Muller Hinton agar media (Oxoid, UK) were used. Sterility of the medium was checked by incubating plates at 37°C for 24 hours. No bacterial growth indicates sterility of the medium. Identification of the bacterial isolates were carried out by standard morphological and biochemical tests including catalase and oxidase test. Apart from this, *P. aeruginosa* also produce certain pigments, non-fluorescent bluish pigment "Pyocyanin", greenish pigment "Pyoverdin", and a red color pigment "pyorubin", which potentially indicates growth of *P. aeruginosa* on agar medium.

Statistical analysis

Statistical analyses were carried out using R software (version 3.2.5, The R Foundation for Statistical Computing). A descriptive summary was presented. Categorical variables were summarised as proportions. Univariable and multivariable logistic regression models were used to model the risk of resistance to Imipenem and Meropenem. Covariates examined were gender, ward, and sample. Gender and ward were always included in the multivariable model as they were of our significance interest.

Antibiotic sensitivity testing

Antibiotic sensitivity testing by Kirby Bauer method on Muller Hinton Agar (MHA) plates by disk diffusion method according to the recommendations by Clinical and laboratory standards institute (CLSI). Antibiotics that were tested include Piperacillin/Tazobactam, Cefoperazone/ sulbactam, ceftazidime, Aztreonam, Cefepime, Amikacin, Ciprofloxacin, Imipenem, Polymyxin B (Table 1).

Metallo-β-Lactamase's testing

EDTA disk synergy test was performed to identify Metallo-β-Lactamase production among *P. aeruginosa*. As previously described by (Yong *et al.*, 2002), that is, "an overnight broth culture of the test strain with opacity adjusted to 0.5 McFarland standard was inoculated onto plates of Mueller-Hinton agar (MHA) as recommended by Clinical and laboratory standards institute (CLSI). A 0.5 M EDTA solution was prepared by dissolving 186 g of disodium EDTA $2H_2O$ in 1,000 ml of distilled water and adjusted it to pH 8.0 by using NaOH.

The mixture was being sterilized by autoclaving. Two 10 µg-Imipenem discs were placed on the plate and appropriate amounts of (10 µl) EDTA solution was added to one of them. The inhibition zones of the Imipenem and Imipenem-EDTA disks were compared after 16 to 18 h of incubation in air at 35°C. Presence of enlarged zone (cut of ≥7 mm) of inhibition was interpreted as EDTA synergy positive, hence MBL positive (Fig. 1).

Results

In present study a total number of 245 *P. aeruginosa* strains were isolated from admitted and attending (OPD) patients from August 2015 to May 2016, at Department of Microbiology, Lady Reading Hospital Peshawar. Out of these, 140 (57.1%) were male patients, while female patients have a number of 105 in total (42.8%). Number of *P. aeruginosa* isolates from outdoor patients were 152 (62%), and from admitted patients were 93 (37%). The majority of the patients were from OPD (62%; 152/245). Burn unit also showed highest number of *P. aeruginosa* isolates (8.16%), followed by Endocrinology (7.75%), surgical (6.93%), and orthopedics (3.25%) respectively (Fig. 2) (Table 2).

Maximum number of *P. aeruginosa* strains were isolated from wound pus (97.14%), followed by ear swab (2.04%), and high vaginal swab (HVS) (1.6%) (Fig. 3) (Table 2). Among 245 isolates of *P. aeruginosa*, 15.1% showed resistance towards Imipenem by Kirby Bauer disk diffusion method. Thirty two (13%) isolates showed MBL production by Imipenem EDTA combined disk test method. Five isolates were negative for MBL production by the said method (Fig.4).

Table 1. Symbols and concentration of antibiotics used.

Antibiotic	Symbol	Concentration per disc
Piperacillin/ Tazobactam	TZP	100 µg/10 µg
Cefoperazone/ Sulbactam	SCF	75 µg/ 10 µg
Aztreonam	ATM	30 µg
Cefepime	FEP	30 µg
Amikacin	AK	10 µg
Ciprofloxacin	CIP	5 µg
Polymyxin B	PB	300 units
Imipenem	IMP	10 µg

Furthermore, females had higher risk of resistance to Imipenem and Meropenem than male (AOR: 1.94; 95%CI: 0.96- 3.93). Patients admitted to Burn unit were at higher risk of resistance to Imipenem and Meropenem than OPD (AOR: 8.07; 95 CI: 2.97- 21.9) (Table 3).

Table 2. Baseline and demographic characteristics.

Gender	N evaluated	n (%)
Female	245	105 (42.8)
Male	245	140 (57.1)
Sample		
Pus	245	238 (97.1)
Ear swab	245	5 (2.04)
High vaginal swab	245	4 (1.6)
Urine	245	2 (0.8)
Sputum	245	1 (0.4)
Sensitivity to imipenem		
Resistant	245	37 (15.1)
Sensitive	245	208 (84.9)
Ward/ Units		
Female skin	245	4 (1.6)
B block	245	1 (0.4)
Burn	245	20 (8.2)
Children	245	2 (0.8)
ENDO	245	19 (7.8)
ENT	245	5 (2)
FCT-4	245	1 (0.4)
Female orthopedic	245	1 (0.4)
Female surgical	245	7 (2.9)
Female urology	245	2 (0.8)
Gynae	245	7 (2.9)
Gynae labor room	245	1 (0.4)
Male medical	245	2 (0.8)
Male orthopedic	245	7 (2.9)
Male surgical	245	10 (4.1)
Male urology	245	3 (1.2)
MCT	245	1 (0.4)
OPD	245	152 (62)

Out of 32 MBL positive isolates, 10 isolates (31%) were from Burn ward, 3 (9%) from Gynecology, 2 (6%) from Endocrinology, and 1 (3%) from surgical ward. Rest of MBL positive isolates were obtained from outdoor patients (n=16, 50%) respectively. Predominate source of all these MBL positive isolates was Pus swab. MBL producers showed 100% resistance to Imipenem, Cefepime, Aztreonam, whereas 100% sensitivity was shown to Polymyxin B, 18% to Piperacillin/ Tazobactam, 9.37% to Ciprofloxacin, and 6.25% to Amikacin & Cefoperazone/ Sulbactam each (Table 4).

Table 3. Risk factors for resistance: Univariable logistic regression and multivariable logistic regression.

	Univariable logistic regression			Multivariable logistic regression		
	(%) [1]Npos/Nobs	OR (95% CI)	P-value	(%) [1]Npos/ Nobs	AOR (95% CI)	P-value
			Gender			
Female	20% (21/105)	1.94 (0.96- 3.93)	0.067	20% (21/ 105)	1.53 (0.69- 3.4)	0.296
Male	11% (16/140)	Reference		11% (16/140)	Reference	
			Ward			
Burn	55% (11/20)	8.07 (2.97- 21.9)	<0.000	55% (11/20)	7.88 (2.89- 21.5)	<0.000
ENDO	11% (2/19)	0.78 (0.17- 3.62)	0.747	11% (2/19)	0.74 (0.16- 3.45)	0.696
Female surgical	14% (1/7)	1.1 (0.13- 9.62)	0.931	14% (1/7)	0.91 (0.1- 8.22)	0.936
Gynae	43% (3/7)	4.95 (1.03- 23.77)	0.046	43% (3/7)	3.9 (0.77- 19.78)	0.100
[2]Other ward	0% (0/40)	No resistance case		0% (0/40)		
OPD	13% (20/152)	Reference		13% (20/152)		
			Sample			
HVS	50% (2/4)	5.8 (0.79- 42.54)	0.084			
Sputum	0% (0/1)	No resistance case				
Urine	0% (0/2)	No resistance case				
Pus	15% (35/238)	Reference				

Discussion

Production of Metallo-β-Lactamase (MBL) by *Pseudomonas aeruginosa* and certain other Gram-negative bacteria has major effect on patient's health. Since these microorganisms carry multi drug resistant genes, and very little therapeutic options are left for physicians to prescribe against these pathogens. Only viable option remains the administration of polymyxin B and Colistin, which are potentially toxic for health. Therefore, detention of MBL producing *P. aeruginosa* is essential to prevent dissémination of infection.

Table 4. Sensitivity pattern of MBL positive isolates.

Antibiotics	MBL positive isolates (n=32)	Percentage %
Piperacillin/ Tazobactam	6	18
Cefoperazone/Sulbactam	2	6.25
Aztreonam	0	0
Cefepime	0	0
Amikacin	2	6.25
Ciprofloxacin	3	9.37
Imipenem	0	0
Polymyxin B	32	100

[1]Npos= number of patients with resistance, Nobs= number of patients for each variable/levels of factors; AOR= Adjusted odds ratio; [2]other ward= Female SKIN B BLOCK, Children, ENT, FCT-4, Female orthopaedic, Female SKIN, Female urology, GYNAE Labour room, Male medical, Male orthopaedic, Male surgical, Male urology, MCT.

In a study carried out by Hemlatha *et al.*, showed that 16% of *P. aeruginosa* isolates were resistant to Imipenem and 14% were positive for MBL production by combined disk test (CDT) (Hemalatha *et al.*, 2005). In 2008 Behera *et al.* reported 10% MBL positive isolates among 14.4% Imipenem resistant *P. aeruginosa* isolates *(Bashir et al.*, 2011).

Fig. 1. Zone of Inhibition of EDTA added imipenem disk, while Imipenem disk alone not showing any zone of inhibition.

In our study, out of 245 isolates of *P. aeruginosa*, 37 (15%) were resistant to Imipenem by Kirby Bauer disk diffusion method. Thirty two (13%) isolates showed MBL production by Imipenem EDTA combined disk test method. Saderi *et al.* from Iran reported much higher percentage of MBL production (94%) in Imipenem resistant *P. aeruginosa* isolates by Imipenem - EDTA combined disc test (CDT) (Saderi *et al.*, 2010). The higher prevalence of MBL positive isolates in the said study may be due to the fact that sampling of isolates was preformed from the burn ward of Hospital. In our study, the highest percentage (31%) were from Burn ward. Burn patients are associated with multiple risk factors, that includes prolong hospital stay, graft application, and surgical intervention which are significant cause of MBL production (Kumar *et al.*, 2012).

MBL's are not only resistant to Carbapenem's but to other medically important antibiotics, that includes aminoglycosides, quinolonas, and cephalosporins (Walsh *et al.*, 2005). In this study only 18% susceptibility was shown by Piperacillin/Tazobactam, 6.25% by Cefoperazone/ Sulbactam and Amikacin, 9.37% by Ciprofloxacin. Polymyxins showed 100% susceptibility, but it cannot be prescribed as mono therapy (Walsh *et al.*, 2005). It can be combined with an appropriate aminoglycoside.

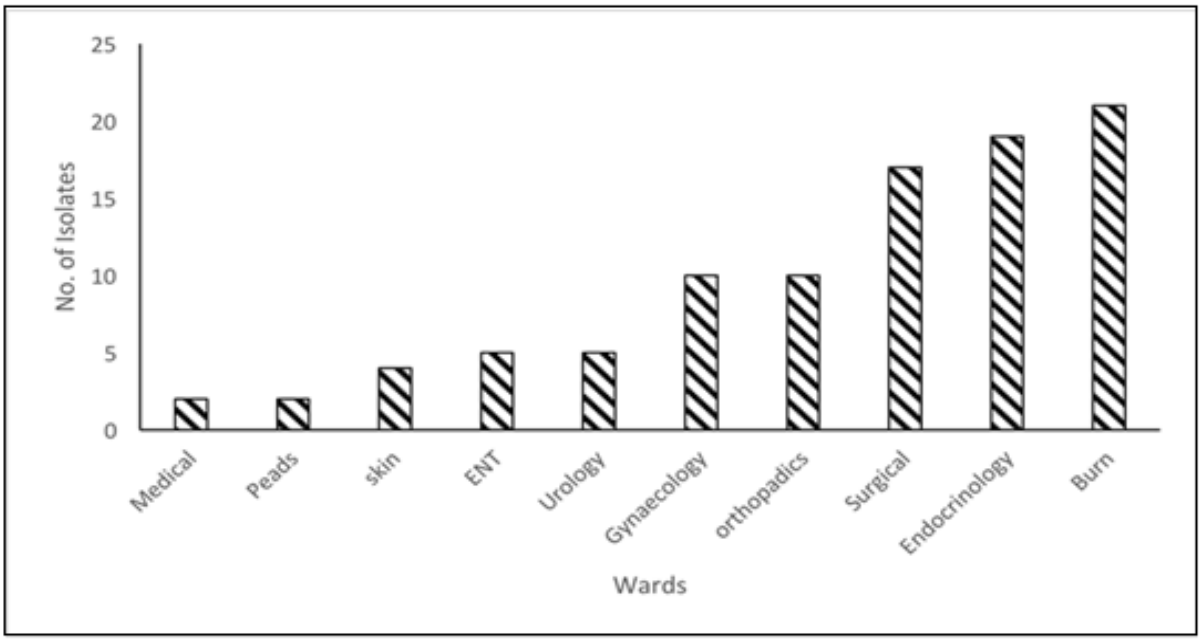

Fig. 2. Number of isolates from different clinical units of Lady Reading Hospital.

Prevalence of MBL positive *P. aeruginosa* in Lady Reading Hospital was alarming. 15% (37 out of 245) isolates were resistant to Imipenem by Kirby Bauer disk diffusion method. 13% (32) isolates showed MBL production by Imipenem-EDTA combined disc test method. Five Imipenem resistant MBL negative isolates might have some other resistance mechanism such as efflux pumps, modified porins. Burn patients showed highest percentage of MBL isolates, suggesting that these patients were most exposed to infections because of tissue grafting or surgical or medical device intervention.

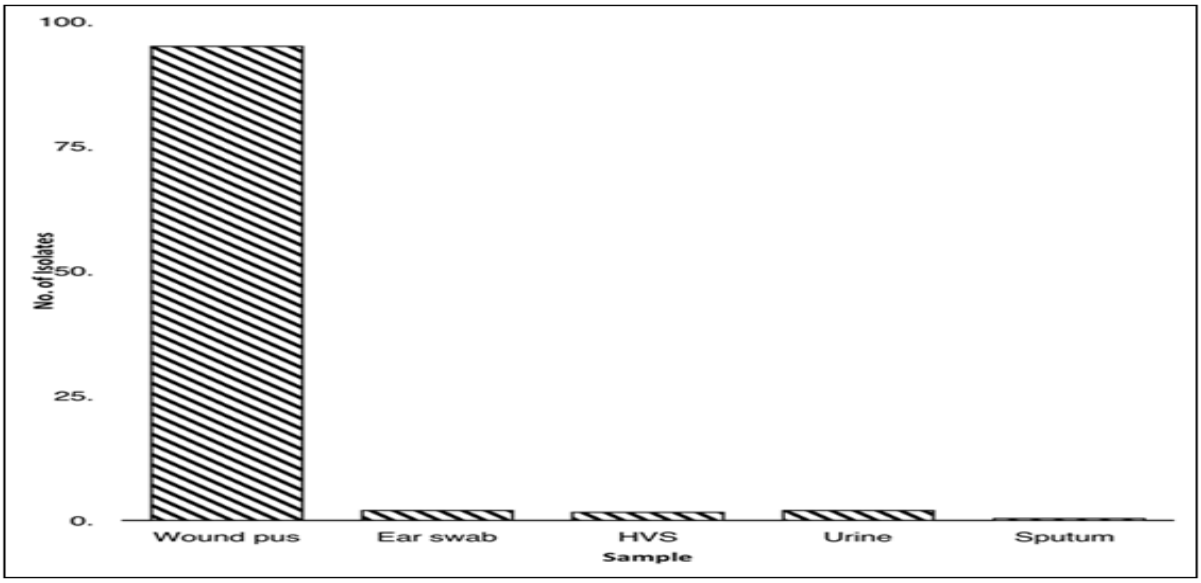

Fig. 3. Percentage of the source of *Pseudomonas* samples.

Fig. 4. Total number of isolates, Imipenem resistant isolates and MBL positive isolates by EDTA- Imipenem combine disk method.

MBL positive isolates showed resistance to almost all beta lactam drugs, while 100% susceptibility was shown to Polymyxin B, making it a key drug to be used against MBL positive isolates. 95% of *P. aeruginosa* isolates were reported from pus samples. Patients from which pus samples were collected mostly have under gone surgical therapies and had prolong hospital stay, which due to improper wound care develop pus making ideal habitat for *P. aeroginosa* to live. 43% of female and 57% male

patients showed *P. aeruginosa* presence, bacteria have potential to cause infection irrespective of gender.

Routine detection of resistance mechanisms such as MBL is essential for clinical laboratories because inappropriate detection can result in treatment failures. Therefore, a reliable and cheap phenotypic detection procedure is required to screen these resistant bacteria in clinical laboratories. Strict antibiotics policy is also a need of the day to present further spread of MBL genes. MBL pose a therapeutic challenge to Clinicians, infection control Pharmacists, and Microbiologists, because most of these microorganisms are multi drug resistant. Implementation of infection control practices not only reduce, but also eliminate and prevent colonization of antibiotic resistant organisms and prevent cross contamination.

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