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Original Research Article

Incidence of Metallo-β-Lactamase Producing Pseudomonas Aeruginosa Isolates and Their Antimicrobial Susceptibility Pattern in Clinical Samples from a Tertiary Care Hospital

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ABSTRACT

Background and objectives: Pseudomonas aeruginosa, most common pathogen causing nosocomial infections, acquire drug resistance to more than one antimicrobial class along with production of metallo-β lactamases (MBL) is one of the most worrisome resistance mechanism. The aim of our study is to find the incidence of MBL in Pseudomonas aeruginosa isolates and their antimicrobial susceptibility pattern.
Materials and Methods: The study included 127 non-repetitive clinical isolates of Pseudomonas aeruginosa from various samples isolated from October 2010 to October 2011, in department of Microbiology, Mahatma Gandhi Medical College and Hospital, Jaipur, Rajasthan. The following tests were used - Imipenem and EDTA combined disc test, Double disc synergy test (DDST) using Imipenem and EDTA and MBL E-test along with antibiotic susceptibility testing.
Result: The incidence of MBL P. aeruginosa was 15/127 (11.81%) in our study. The incidence of MBL pseudomonas was maximum in ICU patients (30%), followed by IPD patients (9.6%). None of the OPD sample grew MBL in Pseudomonas.
Conclusion: Combined disc / DDST using ceftazidime have other ceftazidime resistance mechanisms; will not show MBL production and therefore, imipenem disc should be used for screening of MBL.

Key Words: Pseudomonas aeruginosa, MBL, resistance, carbapenam, imipenam, EDTA

INTRODUCTION

Pseudomonas aeruginosa, one of the most common pathogen, responsible for outbreaks of nosocomial infection in different parts of the world, is responsible for serious infections difficult to treat such as septicemia and pneumonia. Isolates producing ESBL remain sensitive to carbapenems, after the discovery of carbapenemase producing isolates which are resistant to all antibiotics except colistin, polymyxin B & tigecyclin, acts as a precursor for an era of untreatable condition. [1] Resistance to Carbapenem is predominantly mediated by MBL, Ambler molecular class B type of betalactamases that recognize bivalent metal ions, having capacity to hydrolyze all β lactams including carbapenems. [2]

The IMP and VIM genes, responsible for MBL production, transfered horizontally via plasmids, can rapidly

spread to other bacteria, resistant to theraupeutic serine β lactamases inhibitors (such as clavulanate and sulfones). In recent years, MBL gene has spread from Pseudomonas aeruginosa to members of enterobacteriaceae and a clinical scenario simulate the global spread of MBL. [3]

In the absence of novel agents for the treatment of infection caused by multi-drug resistant gram negative bacteria, the uncontrolled spread of MBL producers may lead to treatment failures, with increased morbidity and mortality. So this mechanism of antibiotic resistance among bacterial pathogens is a matter of concern with regard to the future of antimicrobial chemotherapy.

Considering that there is paucity of data regarding MBL Pseudomonas aeruginosa incidence in India we planned our study on "Incidence of Metallo-β-lactamase producing Pseudomonas aeruginosa isolates and their antimicrobial susceptibility pattern in clinical samples" from our tertiary care hospital.

MATERIALS AND METHODS

The present study was conducted on Pseudomonas aeruginosa isolates from October 2010 to October 2011, in department of Microbiology, Mahatma Gandhi Medical College and Hospital, Jaipur, Rajasthan from culture specimen received from different wards, intensive care units and outpatient departments. Clinical samples includes blood, CSF, urine, respiratory secretions, swab from non healing ulcers, pus/wound swab and all other sterile body fluids. These samples except blood & urine were cultured on Blood agar, MacConkey's agar and Thioglycolate broth. Blood was cultured on Brain Heart Infusion broth. Urine was cultured on Blood & MacConkey's agar. All culture plates were incubated overnight at 37°C. Identification of organisms was done by standard laboratory technique based on colony characteristics, Gram′s staining and biochemical tests. [4]

Cultures positive for Pseudomonas aeruginosa were separated out and become a part of our study. They were then subjected to antibiotics sensitivity testing on Muller–Hinton agar plates with commercially available discs (Hi-media, Mumbai) by Modified Kirby Bauer disc diffusion method and were interpreted as per CLSI [5] recommendations using antibiotics (Table 1 & 2) Aminoglycosides [Amikacin (30 µg), Gentamicin (10 µg), Tobramicin (10 µg), Netilmycin (30 µg)], Cephalosporin's [Ceftazidime (30 µg), Ceftriaxone (30 µg), Ceftazidime clavulanic acid, Ceftriaxone salbactam (30 µg)], Floroquinolones [Levofloxacin (10 µg), Ofloxacin (5 µg)], Carbapenems [Imipenem (10 µg)], Penicillins [Ampicillin – clavulanic acid (20/10 µg), Ampicillin – sulbactum (10/10 µg), Piperacillin (100 µg), Piperacillin - tazobactum (100/10 µg), Ticarcillin (75 µg), Ticarcillin – clavulanic acid (75/10 µg)], Aztreonam (30 µg), Macrolide [Tigecyclin (15 µg), Azithromycin (15 µg)] and Tetracyclins [Doxycyclin (30 µg)].

Identification of MBL producing P. aeruginosa: MBL producing *P. aeruginosa* was suspected when the isolates were found resistant to imipenem. We used three methods to identify MBL –

1. Imipenem and EDTA combined disc test: An overnight broth culture of the test strain, opacity finally adjusted to 0.5 McFarland, was used to inoculate a plate of Mueller-Hinton agar. After drying, two imipenem discs (10 µg), placed 20 mm apart from center to center and add 10 µl of 0.5 M EDTA solution to one of the disc. After overnight incubation, increase in zone of inhibition ≥7 mm with the imipenem/EDTA disc as compared to imipenem disc alone, was considered as MBL positive.

To make 0.5 M EDTA solution, 186.1 gm of disodium EDTA was dissolved in 1000 ml of distilled water and pH was adjusted to 8.0 by using NaOH. The mixture was then sterilized by autoclaving.

2. Double disc synergy test (DDST) using Imipenem and EDTA: Test organisms were inoculated on Mueller Hinton agar plates as recommended by the CLSI. An

imipenem (10 µg) disc was placed 20 mm centre to centre from a blank disc containing 10 µl of 0.5 M EDTA solution. After overnight incubation, enhancement of zone of inhibition between imipenem and EDTA disc in comparison with the zone of inhibition of imipenam was interpreted as positive.

3. MBL E-test: For MIC detection of imipenem, the E -test strip method is used. The E test MBL-strip (Biomerieux) contain a double sided seven dilution range of imepenem (IMP 4 to 256 µg/ml) gradient at one end & imipenem (1 to 64 µg/ml) in combination with a fixed concentration of EDTA at the other end. Mueller-Hinton agar plates were inoculated with the isolates equivalent to 0.5 McFarland standards, read after 24 hrs of incubation. If ratio of the MIC of Imepenem/ Imepenem-EDTA was >8 dilutions, it considered as an indicator of MBL production (Figure 1 & Table 3).

Control: β lactamase negative P. aeruginosa ATCC 27853 strains were used as control.

RESULTS

The present study was conducted on 127 non-repetitive clinical isolates of Pseudomonas aeruginosa from various clinical samples. Seventy percent of patients were male, while 30% were females. Maximum number of samples were obtained from Indoor patient department (73.22%) followed by Intensive care units (15.74%) and outdoor patient department (11.02%). Ear swabs were maximum in number (31.5%) followed by sputum (20.5%), pus (18.9%), urine (11%), endotracheal tip (11%), peritoneal fluid (2.36%), blood (0.8%), semen, antral puncture and stool in that order. Maximum samples of endotracheal tip were collected from ICU patients as expected.

The incidence of MBL pseudomonas was maximum in ICU patients (30%), followed by IPD patients (9.6%). None of the OPD sample grew MBL pseudomonas. The antibiotic susceptibility pattern was shown in Figure 2.

Total 15 Pseudomonas aeruginosa samples were found to be Metallo beta lactamase producers and their comparison is shown in Table 4 & 5 by various methods. Out of fifteen MBL samples, 12 were from IPD and 3 were from ICU. None of these MBL Pseudomonas aeruginosa was from OPD. Maximum MBL pseudomonas grew from pus, followed by urine and then endotracheal tube tip. One sample from each of sputum, peritoneal fluid and pouch of douglous grew MBL in pseudomonas.

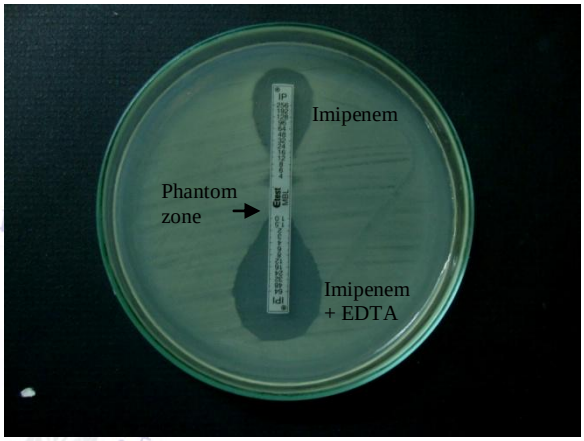

Figure 1: MBL E test: MIC ratio of IP (Imipenem) / IPI (Imipenem-EDTA) of >8 dilutions indicates MBL production. Arrow points towards phantom zone between imipenem and imipenem and EDTA

Table 1: Antimicrobial sensitivity pattern of Pseudomonas aeruginosa isolates

Antimicrobial Agent	Sensitivity (%)
Polymyxin B	127 (100%)
Colistin	127 (100%)
Imipenam	112 (89%)
Piperacillin + Tazobactum	105 (83%)
Tigicyclin	88 (69%)
Azithromycin	84 (66%)
Levofloxacin	80 (63%)
Ceftazidime+clavulanic acid	78 (61%)
Azetronam	80 (63%)
Gentamycin	80 (58%)
Netilmycin	73(57%)
Piperacillin	80 (63%)
Amikacin	71 (56%)
Ceftriaxon+salbactum	69 (54%)
Ceftazidime	63 (50%)
Ofloxacin	62 (48.81%)
Tobramycin	61 (48%)
Cefotaxime	43 (33%)
Ampicillin+salbactum	41 (32%)
Ticarcillin - Clavulanic acid	31 (24%)
Cotrimoxazole	22 (17%)
Ticarcillin	19 (15%)
Doxycycline	9 (7%)

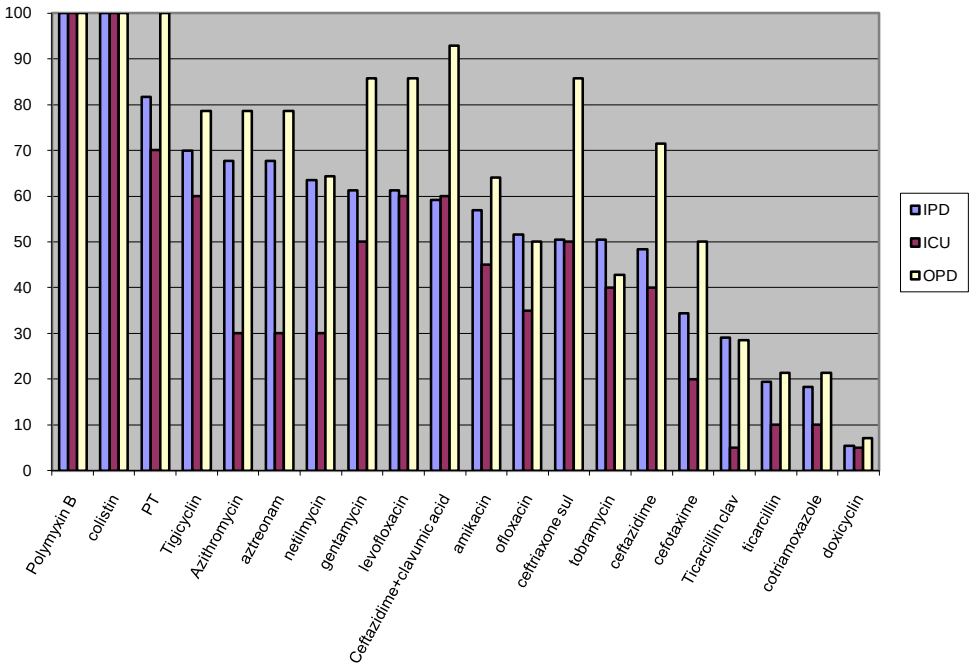

Figure 2: Antimicrobial sensitivity pattern of Pseudomonas aeruginosa isolated from OPD, IPD and ICU

Table 2: Antimicrobial resistance pattern among MBL producers and non MBL producers

Antimicrobial agent	MBL producers (15)		Non MBL producers (112)	
	Total number	%	Total number	%
Amikacin	11	73	45	40
Gentamycin	14	93	33	29
Ampicillin + Sulbactum	14	93	72	49
Piperacillin + Tazobactum	08	53	14	8.9
Ofloxacin	13	86	52	46
Tobramycin	13	86	53	47
Levofloxacin	11	73	36	32
Cotrimoxazole	14	93	91	81
Azithromycin	12	80	31	28
Imipenem	15	100	0	0
Cefotaxime	14	93	70	62
Ceftazidime+ Clavulanic acid	13	86	36	32
Ceftazidime	13	86	51	46
Tigecyclin	03	80	36	32
Netilmycin	14	93	40	36
Azetronam	13	86	34	30

Table 3: MIC (microgram/ml) of imipenem and imipenem+EDTA combination for MBL + strains on E test

S. No.	Imipenem	Imipenem + EDTA
1	48	<1
2	48	<1
3	16	<1
4	48	<1
5	48	<1
6	16	<1
7	32	<1
8	48	<1
9	12	<1
10	12	<1
11	12	<1
12	16	<1
13	64	<1
14	8	<1
15	16	<1

Table 4: Comparison of test results of double disc synergy test, combined disc test and E-test for MBL production (Total Imipenem resistant strains = 15)

Test	MBL producer	%age
Double disc synergy test	10	67%
Combined test test	15	100
E-test	15	100

Table 5: Comparison of different tests for detection of MBL production

Test / Studies	Double disc synergy test	Combined test	E test
Our study	67.67	100	100
B Behra et al	64.28	85.7	100
Deeba Bashir et al	-	86.84	100
Hemlatha et al	-	87.5	-

DISCUSSION

In spite of the advances in antimicrobial agents available for treating critically infected patients, nosocomial infections and their complications caused by *Pseudomonas aeruginosa* act as a major cause of morbidity, mortality and financial burdens worldwide in hospital setting.

Although several studies [6,7] have made available various aspects of the epidemiology of nosocomial infections caused by *Pseudomonas aeruginosa*, till date not much has been done on tracking resistance pattern in nosocomial infection.

Pseudomonas aeruginosa, have limited susceptibility to antimicrobials; a fact corroborated by several research findings over the years [8] that's why overall drug resistance in the world constitutes a serious medical disaster. [9]

There are strong indications that carbepenems (imipenems etc.) are drugs of choice for Multi- drug resistant *Pseudomonas aeruginosa* [9,10] but since from the last few years, substantial amounts of attention on the resistance pattern of these drugs are to be made.

Carbapenems, β-lactam antibiotics, most potent agent for the treatment of multidrug resistant gram negative bacterial infections, have stability against the majority of β-lactamases, have high rate of permeation through bacterial outer membranes. Resistance to carbapenems in Pseudomonas aeruginosa may develop due to impermeability which occurs due to the loss of the opr D porins, up regulation of an active efflux system, or the productions of MBLs.

Carbapenem hydrolyzing MBLs emerged as the most important mechanism of carbapenem resistance, have been reported in many countries. Tremendous therapeutic consequences occurs due to production of MBL by Pseudomonas aeruginosa and other gram negative bacteria, as they also carry other drug resistance genes and the only treatment option available would be like Polymyxin B and Colistin (potentially toxic drugs).

Amongst the MBL positive and negative isolates, maximum sensitivity (53%) was observed for piperacillin/ tazobactam combination as compared to a study conducted by Varaiya A et al, [10] pipercillin / tazobactum had the maximum in vitro susceptibility among other drugs. In fact, combination of antibiotics may be a pitfall in overuse and emergence of drug resistance. Combination therapy, should sufficiently cover relevant pathogens for optimum effect, and minimize the risk of emergence of drug resistance.

However, increasing reports of resistance to carbapenams in Pseudomonas aeruginosa was found in the last decade. The prevalence of MBL positive strain varies from place to place and can be correlated with various factors, like nature of samples, age group of patients, duration of hospitalization, multidrug resistance etc.

The incidence of MBL P. aeruginosa was 11.81% in our study. The incidence of MBL Pseudomonas aeruginsa varies from 5.21% to 69.5% in various studies [11-19] from 2002 to 2011 in Indian region. The difference in incidence is mainly because of difference in patient profile.

Table 6: Incidence of MBL P. aeruginosa

S. No.	Author	Year	Total P. aeruginosa	MBL (%) P. aeruginosa	Reference
	Navaneeth BV et al	2002	50	6 (12%)	[11]
	P Gladstone et al	2003	460 (NFGNB)	24 (5.21)	[12]
	Hemalatha et al	2005	50	8 (16)	[13]
	Shashikala et al	2006	266	29 (10.9)	[14]
	Ami Varaiya et al	2008	240	50 (20.8)	[10]
	Ami Varaiya et al	2008	230	60 (26)	[15]
	A Manoharan et al	2010	61	26 (42.6)	[16]
	Madhu sharma et al	2010	961	668 (69.5)	[17]
	Deeba Bashir et al	2011	283	33 (11.66)	[18]
	Chickmagalure SV et al	2011	54	9 (16.66)	[19]
	Present study	2011	127	15 (11.81)	

The incidence of MBL is very high, in ICU settings, in immuno-compromised patients and in neonates, while it is less when the samples are from OPDs. In a study by Madhu sharma et al, [17] which dealt with neonatal sepsis, the incidence of MBL was as high as 69.5%. Though the percentage of MBL P. aeruginosa among total samples from ICU patients was less (5.21%) in Gladstone study, [12] it was because, the incidence was calculated as a percentage of total Non fermenting Gram negative bacilli and not of total P aeruginosa strains.

There is no guideline for MBL detection, various studies have reported the use of different methods like imipenem-EDTA combined disc test, double-disc synergy test using imipenem and EDTA, E test and modified Hodge.

Recently, CLSI has recommended modified Hodge test for carbapenemases activity in Enterobacteriaceae, but not in non-fermenters. In our study we used three different methods for detection of MBL producing P. aeruginosa; Imipenem plus EDTA combination disc test, Double Disc Synergy test and E-test. A total of 127 strains were isolated over a study period of one year. Fifteen (11.18%) out of 127 were resistant to imipenem and showed a significant zone enhancement of >7 mm around imipenem-EDTA combination disc in comparison to imipenem disc alone, thus confirming their MBL status.

All the fifteen isolates on which E test [20] was performed were also MBL positive. Thus 100% positive results were obtained in our study which is comparable to various studies. Both combined disc and E-test were found to be equally sensitive in detecting MBL positive isolates. Considering the cost constraints of E-test, a simple screening test like combined imipenem / imipenem + EDTA method can be used to find MBL positive isolates in various clinical specimens, as the technique is very easy, economical and can be incorporated into the routine testing of any busy microbiological laboratory, as has been done for ESBL screening.

We also performed DDST on these 15 imipenem resistant Pseudomonas aeruginosa samples. Ten out of fifteen (66.7%) strains showed enhancement of zone of inhibition in the area between imipenem and EDTA disc in comparison with the zone of inhibition of imipenam. B Behra et al [21] also showed decreased sensitivity of DDST in comparison with combined disc test, out of 56 isolated strains, 48 exhibited a zone enhancement of ≥7 mm in the combined disc test whereas only 36 isolates gave positive result by DDST. One of the major disadvantages of DDST is the subjective interpretation of results.

CONCLUSION

Although some authors recommend ceftazidime disc instead of imipenem, combined disc / DDST using ceftazidime will not be able to show MBL production as they have different type of ceftazidime resistance mechanisms and therefore, imipenem disc must be used as a screening tool for detection of MBL production. Also there is a need of regular consideration for detection of MBL producers in microbiology lab on routine basis and effective use of antimicrobials to halt the emergence of resistant organisms.

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