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Phenotypic Evaluation of Prevalence of Metallo-Beta-Lactamase (MBL) Production among Clinical Isolates of *Pseudomonas aeruginosa* and *Acinetobacter* Species in a Tertiary Care Hospital of North India

Noor Jahan[1], Razia Khatoon[2]* and Mohd Rashid[3]

[1]Department of Microbiology, Integral Institute of Medical Sciences and Research, Integral University, Lucknow-226026, India
[2]Department of Microbiology, Hind Institute of Medical Sciences, Mau, Ataria, Sitapur-261303, India
[3]Department of Microbiology, F.H. Medical College, Tundla, Agra-283204, India

**Corresponding author*

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A B S T R A C T

Pseudomonas aeruginosa and *Acinetobacter* species have emerged as important nosocomial pathogens. Carbapenems had been the drugs of choice for penicillin & cephalosporin resistant *Pseudomonas* and *Acinetobacter* species infections. However, this scenario has changed with the emergence of Metallo-beta-lactamase (MBL) producing strains as these enzymes hydrolyze all beta-lactams, thereby, increasing patient morbidity and mortality. This situation prompts early and accurate detection of MBL producers. Hence the present study was done to phenotypically evaluate the prevalence of MBL production among 235 clinical isolates of *Pseudomonas aeruginosa* (183) and *Acinetobacter* species (52). The antimicrobial susceptibility testing was done by Kirby Bauer disk diffusion method. About 26.4% were found to be resistant to carbapenems tested. These screen positive isolates gave results of positive MBL production among 79.0% and 59.7% isolates by imipenem-EDTA combined disk test and modified Hodge test respectively. The prevalence of MBL production was found to be 21.9% and 17.3% among isolates of *Pseudomonas aeruginosa* and *Acinetobacter* species respectively. The prevalence was found to be higher among isolates from inpatients (26.1%) in comparison to those from outpatients (9.5%). Most of the MBL producers were isolated from pus samples (33.7%), followed by sputum (18.6%). The *in vitro* antimicrobial susceptibility profile of MBL producers showed that they were multidrug resistant, being 100% sensitive only to colistin and polymyxin B. To conclude, detection of MBL producers should be routinely done in all microbiological laboratories along with implementation of strict infection control policies and antibiotic stewardship for better patient management.

Introduction

Pseudomonas and *Acinetobacter* species have emerged as important nosocomial pathogens. They are widely distributed in nature and their presence in the hospital environment puts debilitated patients, especially those in intensive care units (ICUs) at risk of

opportunistic infections by these multidrug resistant pathogens (Sarkar *et al.,* 2006). Carbapenems had been the drugs of choice for penicillin and cephalosporin resistant *Pseudomonas* and *Acinetobacter* species infections, because of their broad spectrum activity and stability to hydrolysis by most of the beta-lactamases including extended spectrum beta lactamases (ESBLs). However, this scenario has changed with the emergence of Metallo-beta-lactamase (MBL) producing strains (Varaiya *et al.,* 2008; Ahir *et al.,* 2012; Shivappa *et al.,* 2015).

Metallo-beta-lactamases (MBL) are metalloenzyme of Ambler class B which require divalent cations of zinc as cofactors for enzyme activity and are inhibited by metal chelators like ethylene diamine tetra acetic acid (EDTA) and thiol-based compound but not by sulbactam, tazobactam and clavulanic acid (Purohit *et al.,* 2012). The MBLs efficiently hydrolyze all beta-lactams, except monobactam i.e. aztreonam (Galani *et al.,* 2008). The genes for MBL production (IMP and VIM) are horizontally transferable via plasmids and can rapidly spread to other bacteria (Senda K *et al.,* 1996; Bennett, 1999). Several studies have reported global increase in the prevalence of MBL producing non-fermenting bacilli (Varaiya *et al.,* 2008; Saha R *et al.,* 2010; Deshmukh *et al.,* 2011). MBL production is typically associated with resistance to aminoglycosides and fluoroquinolones, further compromising the therapeutic options (Purohit *et al.,* 2012). Thereby making it a matter of concern with regard to the future of antimicrobial chemotherapy (Bush *et al.*, 1995).

This situation prompts an early and accurate detection of MBL producing organisms of crucial importance. Carbapenemase gene detection by molecular methods is the gold standard, but is available in only few reference laboratories, therefore, phenotypic tests have been developed for detection of MBL producers in clinical laboratories (Andre *et al.,* 2012). Therefore, we did this study to evaluate the prevalence of metallo-beta-lactamase (MBL) producing *Pseudomonas aeruginosa* and *Acinetobacter* species derived from clinical samples at our Tertiary care hospital by using phenotypic methods.

Materials and Methods

A hospital based prospective study was done over a period of 1 year from January to December 2017. The study was approved by Institutional Ethics Committee. Various clinical samples such as pus, urine, blood, sputum and catheter tip, received in bacteriology laboratory of department of Microbiology, from both outpatient departments (OPD) and inpatient departments (IPD including patients admitted in various wards and intensive care units) were cultured on Blood agar and MacConkey agar and incubated aerobically at 37°C for 24 hours and the growth was identified as per the standard microbiological protocols and procedures (Crichton, 2006). A total of 235 consecutive, non-duplicate isolates of *Pseudomonas aeruginosa* (N = 183) and *Acinetobacter* species (N = 52) were included in the study. All gram positive and other Gram negative bacterial isolates were excluded. Antimicrobial susceptibility testing was performed on Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India) by Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines using antibiotics (HiMedia Laboratories, India) such as, amikacin (30µg), gentamicin (10µg), piperacillin (100µg), piperacillin/tazobactam (100/10µg), ampicillin/sulbactam (10/10µg), ceftazidime (30µg), cefotaxime (30µg), ceftriaxone (30µg), cefepime (30µg), imipenem (10µg), meropenem (10µg), colistin (10µg), polymyxin B (300 units), ciprofloxacin (5µg)

and aztreonam (30µg). *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC BAA-1705 (Modified Hodge Test positive) and *Klebsiella pneumoniae* ATCC BAA-1706 (Modified Hodge Test negative) were used as quality control strains (CLSI, 2016).

Screening method for MBL production

As mechanisms of resistance can be different for imipenem and meropenem, therefore, resistance to imipenem is not always predictive of resistance to meropenem, and vice versa (Jones *et al.*, 2006). Hence, isolates resistant to imipenem (zone size ≤ 15 mm for *Pseudomonas aeruginosa* and ≤ 18 mm for *Acinetobacter* species) and / or meropenem (zone size ≤ 15 mm for *Pseudomonas aeruginosa* and ≤ 14 mm for *Acinetobacter* species) were considered as screening positive (CLSI, 2016). These isolates were then subjected to two different phenotypic tests for confirmation i.e. Imipenem-EDTA combined disk test and modified Hodge test.

Imipenem - EDTA Combined disk test (CDT)

One 10 µg imipenem disk alone along with another 10 µg imipenem disk supplemented with 750 mg EDTA (Hi-Media Lab, India) were placed at a distance of 20 mm from center to center on lawn culture of the test organism on Mueller Hinton agar and incubated at 35°C for 16-18 hrs (Altun *et al.*, 2013; El-Din *et al.*, 2014). The inhibition zones of imipenem and imipenem EDTA was compared and if zone of inhibition of imipenem-EDTA disk was ≥ 7 mm more than that of imipenem disk alone, it was considered as MBL producer (Figure 1).

Modified Hodge Test (MHT)

A 0.5 McFarland standard suspension of *Escherichia coli* ATCC 25922 was prepared in broth. The surface of a Mueller Hinton agar plate was inoculated evenly with 1:10 dilution of the suspension in broth using a sterile swab as for the routine disk diffusion procedure. After drying the plate for 15 min imipenem disk (10 µg) was placed at the center of the plate and the imipenem resistant test strains from the overnight culture plates along with the positive (*Klebsiella pneumoniae* ATCC BAA-1705) and negative (*Klebsiella pneumoniae* ATCC BAA-1706) control for MHT were streaked heavily from the edge of the disk to the periphery of the plate (Lee *et al.*, 2001). The presence of a distorted inhibition zone of imipenem after overnight incubation was interpreted as modified Hodge test positive (Figure 2).

Statistical analysis

The collected data were statistically analyzed using SPSS software, Chicago, version 16. The association between MBL production and resistance to antibiotics was analyzed using Chi-square test and p value < 0.05 was considered as statistically significant.

Results and Discussion

A total of 235 isolates of *Pseudomonas aeruginosa* (N = 183) and *Acinetobacter* species (N = 52) isolated from various clinical samples were screened for MBL production as shown in Table 1. This finding was found to be statistically significant ($p < 0.001$). Out of these 235 clinical isolates, 26.4% (62/235) were found to be resistant to carbapenems tested (imipenem and / or meropenem) and hence were screening test positive for MBL production, with 67.7% *Pseudomonas aeruginosa* and 32.3% *Acinetobacter* species as depicted in Figure 3. These screen positive isolates on being subjected to phenotypic confirmatory tests yielded positive MBL production among 79.0% (49/62) and 59.7% (37/62) isolates by CDT and MHT respectively, this difference was found to be

statistically significant ($p < 0.001$) as depicted in Table 2. It was found that the prevalence of MBL production was higher among isolates of *Pseudomonas aeruginosa* (21.9%, 40/183) as compared to those among *Acinetobacter* species (17.3%, 9/52) as shown in Table 3. However, this difference was not found to be statistically significant ($p = 0.476$). Also, the prevalence of MBL producing organisms was found to be higher among isolates from inpatients (26.1%, 42/161) in comparison to those from outpatients (9.5%, 07/74) as depicted in Table 4, this difference was also found to be statistically significant ($p = 0.004$). Table 5 shows that most of the MBL producers were isolated from pus samples (33.7%), followed by sputum (18.6%), and least from blood (4.8%). This finding was found to be statistically significant ($p = 0.002$).

The *in vitro* antimicrobial susceptibility profile of 183 isolates of *Pseudomonas aeruginosa* and 52 isolates of *Acinetobacter* species showed that MBL producers possessed multidrug resistance with highly decreased susceptibility to piperacillin, piperacillin/ tazobactam, ceftazidime, cefepime, amikacin, gentamicin as well as to ciprofloxacin. All the MBL producers were found to be highly resistant to imipenem (100%) and meropenem (100%), and highly sensitive to colistin (100%) and polymyxin B (100%). The susceptibility pattern of MBL producers for most of the tested drugs was found to be statistically significantly different ($p < 0.05$) as compared to non-MBL producers as depicted in Table 6 and 7.

In the present study majority of the isolates were of *Pseudomonas aeruginosa* (77.9%) as compared to *Acinetobacter* species (22.1%). A very high percentage of *Pseudomonas aeruginosa* were isolated from pus samples (91.3%), followed by urine (87.2%) and least from sputum (55.8%), whereas, majority of *Acinetobacter* species were isolated from sputum (44.2%) followed by catheter tip (37.5%), and least from pus (8.7%). However, in contrast to our finding a study from Mysore detected very high percentage of *Pseudomonas* (36%) and *Acinetobacter* (32%) isolated from endotracheal tube suction samples followed by pus samples (11.7% and 4.94% respectively) and very low percentage isolated from urine samples (0.24% and 0.35% respectively) (Shivappa *et al.*, 2015).

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Table.1 Distribution of organisms isolated from various clinical samples (N = 235)

Samples tested	*Pseudomonas aeruginosa*, N (%)	*Acinetobacter* species, N (%)	Chi-Square (χ^2) and *p value
Pus (N = 92)	84 (91.3%)	08 (8.7%)	$\chi^2 = 30.084$, $p < 0.001$
Urine (N = 47)	41 (87.2%)	06 (12.8%)	
Blood (N = 21)	14 (66.7%)	07 (33.3%)	
Sputum (N = 43)	24 (55.8%)	19 (44.2%)	
Catheter Tip (N = 32)	20 (62.5%)	12 (37.5%)	
Total (N = 235)	**183 (77.9%)**	**52 (22.1%)**	

N = Number of isolates. *p value < 0.05 was considered as statistically significant.

Table.2 Comparative evaluation of MBL production among screen positive isolates (N = 62) by using Imipenem-EDTA combined disk test (CDT) and Modified Hodge test (MHT)

CDT	MHT			Chi-Square (χ^2) and *p value
	MBL production present, N (%)	MBL production absent, N (%)	Total isolates, N (%)	
MBL production present, N (%)	37 (75.5%)	12 (24.5%)	**49 (100%)**	χ^2 = 24.344, p < 0.001
MBL production absent, N (%)	0 (0%)	13 (100%)	**13 (100%)**	
Total isolates, N (%)	**37 (59.7%)**	**25 (40.3%)**	**62 (100%)**	

N = Number of isolates. *p value < 0.05 was considered as statistically significant.

Table.3 Distribution of organisms on the basis of their MBL producing status (N = 235)

Organisms	MBL producers, N (%)	Non-MBL producers, N (%)	Total isolates tested N (%)	Chi- Square (χ^2) value and *p value
Pseudomonas aeruginosa	**40 (21.9%)**	**143 (78.1%)**	**183 (100%)**	χ^2 = 0.508, p = 0.476
***Acinetobacter* species**	**09 (17.3%)**	**43 (82.7%)**	**52 (100%)**	
Total isolates	**49 (20.9%)**	**186 (79.1%)**	**235 (100%)**	

N = Number of isolates. * p < 0.05 was considered as statistically significant.

Table.4 Distribution of organisms according to their MBL producing status and source of samples tested (N = 235)

Source of Samples	MBL producers N (%)	Non-MBL producers N (%)	Total isolates N (%)	Chi- Square (χ^2) value and *p value
Inpatients	42 (26.1%)	119 (73.9%)	161 (100%)	χ^2 = 8.493, p = 0.004
Outpatients	07 (09.5%)	67 (90.5%)	74 (100%)	
Total isolates	**49 (20.9%)**	**186 (79.1%)**	**235 (100%)**	

N = Number of isolates. * p < 0.05 was considered as statistically significant.

Table.5 Distribution of MBL producing organisms according to the samples tested (N = 235).

Samples tested	MBL producers N (%)	Non-MBL producers N (%)	Total isolates N (%)	Chi- Square (χ^2) value and *p value
Pus	31 (33.7%)	61 (66.3%)	92 (100%)	χ^2 = 17.489, p = 0.002
Urine	04 (8.5%)	43 (91.5%)	47 (100%)	
Blood	01 (4.8%)	20 (95.2%)	21 (100%)	
Sputum	08 (18.6%)	35 (81.4%)	43 (100%)	
Catheter Tip	05 (15.6%)	27 (84.4%)	32 (100%)	
Total	**49 (20.9%)**	**186 (79.1%)**	**235 (100%)**	

N = Number of isolates. * p < 0.05 was considered as statistically significant.

Table.6 Comparative evaluation of *in vitro* antibiotic susceptibility pattern of MBL producing and non-MBL producing isolates of *Pseudomonas aeruginosa* for the commonly used antibiotics (N = 183)

Antibiotics tested	Percentage of susceptible isolates among MBL producers (N = 40)	Percentage of susceptible isolates among Non-MBL producers (N = 143)	Chi-Square (χ^2) and *p value
Amikacin	15.0%	64.3%	$\chi^2 = 30.586$, $p < 0.001$
Gentamicin	10.0%	54.5%	$\chi^2 = 25.079$, $p < 0.001$
Piperacillin	0%	26.6%	$\chi^2 = 13.415$, $p < 0.001$
Piperacillin-tazobactam	5.0%	74.1%	$\chi^2 = 61.751$, $p < 0.001$
Ceftazidime	0%	18.9%	$\chi^2 = 8.860$, $p = 0.003$
Cefepime	0%	51.7%	$\chi^2 = 34.752$, $p < 0.001$
Aztreonam	30.0%	21.0%	$\chi^2 = 1.438$, $p = 0.230$
Ciprofloxacin	7.5%	41.3%	$\chi^2 = 15.902$, $p < 0.001$
Imipenem	0%	96.5%	$\chi^2 = 156.979$, $p < 0.001$
Meropenem	0%	98.6%	$\chi^2 = 171.848$, $p < 0.001$
Colistin	100%	100%	NA
Polymyxin B	100%	100%	NA

N = Number of isolates. *p value < 0.05 was considered as statistically significant. NA = Not Applicable.

Fig.1 Shows an isolate with zone of inhibition of imipenem-EDTA disk ≥ 7 mm more than that of imipenem disk alone, hence, it was considered as MBL producer

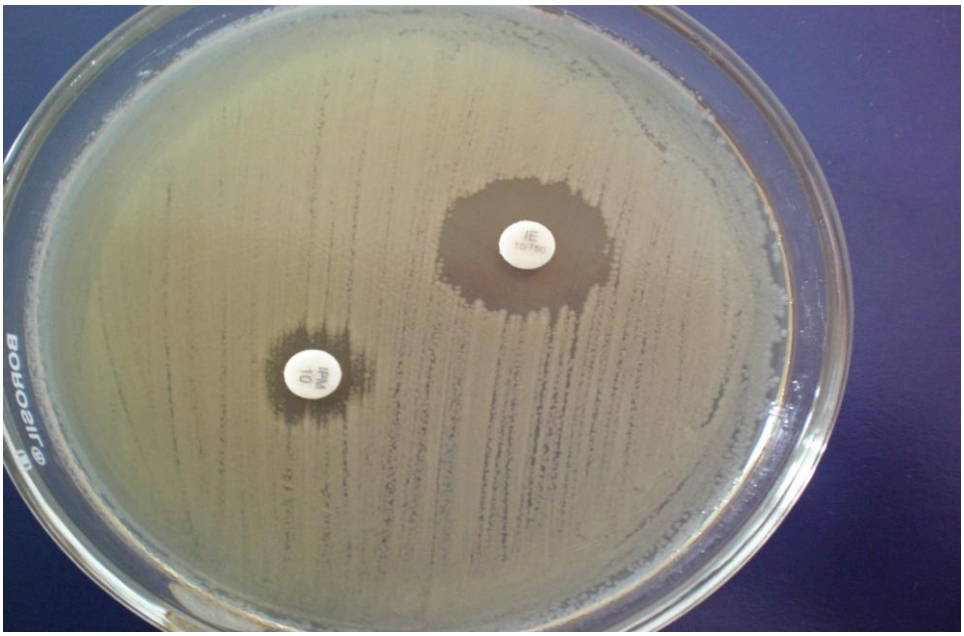


Table.7 *In vitro* antibiotic susceptibility pattern of MBL producing and non-MBL producing isolates of *Acinetobacter* species included in the study (N = 52)

Antibiotics tested	Percentage of susceptible isolates among MBL producers (N = 9)	Percentage of susceptible isolates among Non-MBL producers (N = 43)	Chi-Square (χ^2) and *p value
Ampicillin-sulbactam	33.3%	46.5%	$\chi^2 = 0.524$, p = 0.469
Ceftazidime	0%	16.3%	$\chi^2 = 1.693$, p = 0.193
Cefotaxime	0%	23.3%	$\chi^2 = 2.591$, p = 0.107
Ceftriaxone	0%	25.6%	$\chi^2 = 2.920$, p = 0.087
Cefepime	0%	44.2%	$\chi^2 = 6.266$, p = 0.012
Amikacin	0%	60.5%	$\chi^2 = 10.884$, p = 0.001
Gentamicin	0%	55.8%	$\chi^2 = 9.329$, p = 0.002
Piperacillin	0%	18.6%	$\chi^2 = 1.979$, p = 0.160
Piperacillin-tazobactam	11.1%	65.1%	$\chi^2 = 8.800$, p = 0.003
Ciprofloxacin	11.1%	48.8%	$\chi^2 = 4.340$, p = 0.037
Imipenem	0%	88.4%	$\chi^2 = 29.542$, p < 0.001
Meropenem	0%	90.7%	$\chi^2 = 32.651$, p < 0.001
Colistin[§]	100%	100%	NA
Polymyxin B[§]	100%	100%	NA

N = Number of isolates. *p value < 0.05 was considered as statistically significant. § These antibiotics were tested by agar dilution method for MIC and results ≤ 2µg/ml was taken as sensitive. NA = Not Applicable.

Fig.2 Shows that the test strain produces the enzyme and allows the growth of the carbapenem susceptible *Escherichia coli* ATCC 25922 strain towards the imipenem disk, thereby causing the appearance of distorted inhibition zone of imipenem after overnight incubation, hence, the test strain was interpreted as modified Hodge test positive and an MBL producer

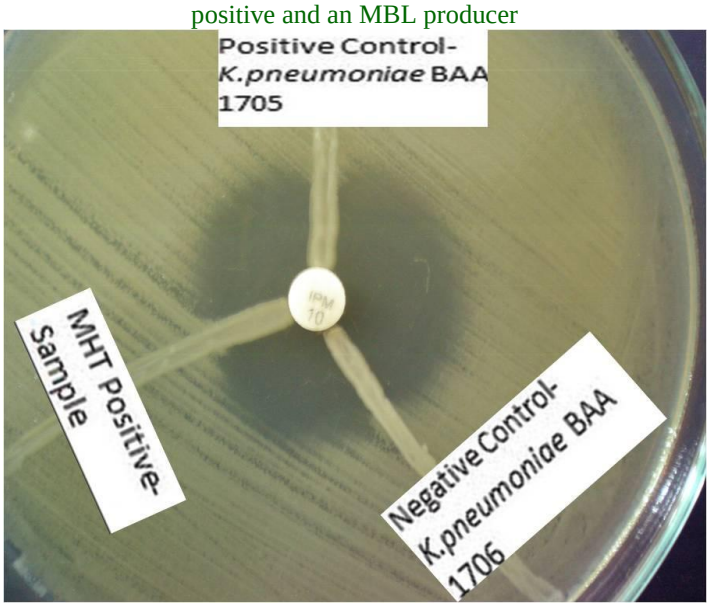
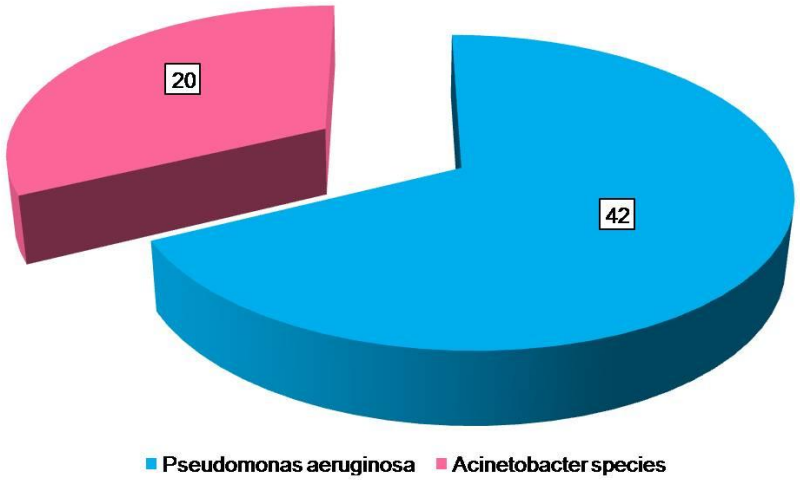


Fig.3 Shows the distribution of isolates of *Pseudomonas aeruginosa* and *Acinetobacter* species resistant to carbapenems tested, hence, they were considered as screen positives for MBL production



In our study, out of 235 isolates, 62 were found to be carbapenem resistant, these isolates when subjected to confirmatory tests yielded positive results of MBL producing organisms among 79.0% isolates by CDT and 59.7% isolates by MHT. Similarly a study done in Meerut detected more number of MBL producers among organisms using CDT as compared to MHT (Chauhan *et al.*, 2015). In our study, the prevalence of MBL production was found to be higher among isolates of *Pseudomonas aeruginosa* (21.9%) as compared to those among *Acinetobacter* species (17.3%). Our this finding is in agreement with another study done in Gujarat which also detected higher prevalence of MBL production among isolates of *Pseudomonas aeruginosa* (11.42%) as compared to those among *Acinetobacter* species (10.40%) (Ahir *et al.*, 2012). Another study done in Kolkata, also detected higher prevalence of MBL production among *Pseudomonas aeruginosa* isolates (41%) as compared to *Acinetobacter* species (22%) (Rit *et al.*, 2013). However, in contrast to our finding, a study from Mumbai detected higher prevalence of MBL production among isolates of *Acinetobacter* species (36%) as compared to *Pseudomonas aeruginosa* (28.57%) (De *et al.*, 2010).

In the present study, the prevalence of MBL producing organisms was found to be higher among isolates from inpatients (26.1%) in comparison to those from outpatients (9.5%). However, detection of MBL production in an organism isolated from samples received from outpatients is a matter of concern as such strains may spread rapidly into the community and cause therapeutic problem.

In our study, most of the MBL producers were isolated from pus samples (33.7%), followed by sputum (18.6%), and least from blood (4.8%). Our this finding corroborates well with another study from Maharashtra which also detected maximum MBL producers isolated from pus samples (36.8%), followed by tracheal secretions (26.3%), urine (15.9%) and least from blood and ascitic fluid (10.6% each) (Deshmukh *et al.*, 2011).

In the present study, antibiotic susceptibility profile showed that MBL producers were

multidrug resistant, with both *Pseudomonas aeruginosa* and *Acinetobacter* species isolates being highly resistant to all the drugs tested except colistin and polymyxin B to which both were still 100% sensitive. This finding corroborates with another study from Kolkata which detected that MBL producing isolates were multidrug resistant except for colistin (100% sensitive) and for polymyxin-B (92% sensitivity by *Pseudomonas aeruginosa* and 85% sensitivity by *Acinetobacter* species) (Rit *et al.,* 2013). Another study from Maharashtra also detected that all the MBL producers were 100% sensitive to colistin (Deshmukh *et al.,* 2011). Similarly, various workers have reported polymyxin B as the most sensitive drug for MBL producers (Pandya *et al.,* 2011). But polymyxin B being a very toxic drug should not be used as a monotherapy. It can be combined with an appropriate aminoglycoside. Aztreonam is the drug of choice for MBL producing *Pseudomonas aeruginosa* (Walsh *et al.,* 2005). Combination therapy is often employed in treatment of multidrug-resistant *Acinetobacter* species (De *et al.,* 2010). Imipenem or meropenem combined with ampicillin-sulbactam is found to be active against carbapenem-resistant as well as MBL-positive strains of *Acinetobacter* species (Perez *et al.,* 2007).

To conclude, our finding shows that there are significant numbers of MBL producing isolates with multidrug resistance not only among hospitalized patients but also among outpatients. This situation prompts the early detection of MBL-producing isolates which would help in reduction of mortality rates of patients and also to avoid the intra-hospital and inter-hospital dissemination of such strains. Detection of MBL is a challenge for routine microbiology laboratories, since there are no standardized methods for MBL detection. However, as detected in our study, CDT (Imipenem-EDTA) is the most convenient phenotypic method for detection of MBL production in Gram negative bacilli with high sensitivity and its advantage is that it is also less time consuming, technically less demanding as compared to MHT, therefore, less cumbersome to perform in routine microbiological laboratories. Also, one must enforce strict infection control policies and antibiotic policies for judicious use of carbapenems and other broad spectrum antibiotics in order to reduce the escalation of such resistant organisms.

Conflict of Interest: None declared.

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