

Phenotypic Assays for Detection of AmpC and MBL Producers among the Clinical Isolates of Multi Drug Resistant *Pseudomonas aeruginosa*

Sarita Manandhar^{1*}, Sarashwoti Adhikari¹, Sujeeb Rajbhandari²

¹Tri Chandra Multiple College, Ghantaghar, Kathmandu, Nepal

²Shahid Gangalal National Heart Center, Bansbari, Kathmandu, Nepal

*Corresponding author: Sarita Manandhar, Tri Chandra Multiple College, Ghantaghar, Kathmandu, Nepal Email: sarita111@gmail.com

ABSTRACT

Objectives: In order to determine the prevalence of multi-drug resistance along with AmpC and metallo- β -lactamase producing *P. aeruginosa*, a six month cross-sectional study was carried out at Shahid Gangalal National Heart Center.

Methods: A total of 756 clinical specimens were analyzed for bacteriological profile. The bacterial isolates were identified by cultural and biochemical techniques. Antibiotic susceptibility testing of the isolates was performed by Kirby-Bauer disc diffusion method. MDR isolates were screened and tested for MBL and AmpC production. Ceftazidime resistant isolates were tested for MBL and Cefoxitin resistant isolates for AmpC.

Results: Among all the clinical samples analyzed, *P. aeruginosa* was detected in 75 samples (9.92%). Antibiotic susceptibility testing showed Imipenem as the most effective drug with susceptibility of 76% followed by Piperacillin-Tazobactam (74.7%) and Piperacillin (41.3%). Out of 75 *P. aeruginosa* isolates, 53 (70.6%) of them were found to be resistant to at least three out of four anti-pseudomonal agents, thus were considered as MDR. Out of 53 multi-drug resistant *P. aeruginosa* (MDRPA), all were resistant to ceftazidime whereas 85% (45/53) were resistant to cefoxitin. Out of 53 isolates, 11 (20.75%) showed positive result for MBL. Similarly, 7 out of 45 i.e. 13.2% were found to be AmpC producers.

Conclusion: This study signified the high prevalence of MDRPA which is an alarming rate. Also multiple β -lactamase producing *P. aeruginosa* were detected which can further complicate the treatment options. Regular monitoring of antibiotic susceptibility and rational use of antibiotics would be helpful in eliminating the outbreaks of multiple β -lactamase producing MDRPA.

Key words: *P. aeruginosa*, β -lactams, MDRPA, AmpC, MBL

INTRODUCTION

Pseudomonas aeruginosa is an increasingly prevalent opportunistic human pathogen and the most common gram-negative bacterium found in nosocomial infections (Kalaivani 2011). Most distinguishing feature of this organism is the capability to confer resistance to several classes of antibiotics using distinctive mechanisms (Kapoor et al. 2011; Tian et al. 2011). Though carbapenems remain one of the best drugs to treat infections caused by *P. aeruginosa*, increasing usage of these drugs and other expanded-spectrum antibiotics has resulted in the development of carbapenem resistant

P. aeruginosa creating therapeutic problem (Yousefi et al. 2010; Martinez et al. 2011). Aztreonam is the only β -lactam antibiotic that is saved from the activity of β -lactamases (Tsakris et al. 2009). The common form of resistance is mediated by lack of drug penetration and/or carbapenem hydrolysing β -lactamase enzymes including the metallo- β -lactamases (MBL) (Shanthi and Sekar 2009; Ishii et al. 2010). Metallo- β -lactamases are a diverse set of enzymes having zinc ion at the active site that catalyze the hydrolysis of a broad range of β -lactam drugs (including carbapenems) with the exception of monobactams. An increasing prevalence of carbapenem

resistance mediated by acquired metallo- β -lactamases (MBLs) is being reported, particularly for *P. aeruginosa* clinical isolates in several countries. The resistance may spread rapidly to various species of gram-negative bacilli, as the MBL genes reside in mobile gene cassettes inserted in integrons. The production of metallo- β -lactamases by bacteria is becoming a serious threat to the clinical community because these enzymes are responsible for the development of antibiotic resistance to the commonly employed β -lactam antibiotics.

Ambler class C, confer resistance to cephamycins, narrow, expanded and broad-spectrum cephalosporins, Aztreonam and β -lactam/ β -lactamase inhibitor combination. The enzyme may be chromosomal or plasmid encoded. Although these two enzymes have similar substrate profile, the only difference is chromosomal AmpC are inducible whereas plasmid mediated AmpC are uninducible.

Despite the discovery of AmpC and Metallo- β -lactamases at least a decade ago, there remains a low level of awareness of their importance and many clinical laboratories have problems in detecting AmpC and metallo- β -lactamase. Failure to detect these enzymes has contributed to their uncontrolled spread and sometimes to therapeutic failures (Singhal et al. 2005).

MATERIALS AND METHODS

This hospital based cross sectional study was carried out from April 2014 to September 2014 in the Microbiology laboratory of Shahid Gangalal National Heart Center. A total of 756 different samples including blood, sputum, urine, pus, endo-tracheal (ET) secretion, wound swab, bed sore swab, Central Venous Pressure (CVP) tip, nasal secretion and oral secretion were collected from the patient and processed following standard laboratory techniques. The samples were inoculated on Blood agar and MacConkey agar and incubated at 37°C for 24 hours. For the identification of *Pseudomonas* spp., typical non-fermenting colonies from MacConkey agar were further processed via gram staining and other biochemical tests. *P. aeruginosa* was identified on the basis of various characteristics such as positive catalase and oxidase test, motile, mannitol non-fermenter, growth at 42°C, oxidative in Hugh and Leifson's medium, indole negative, methyl red/voges proskauer test negative, citrate positive, Alk/no change, H₂S negative, and gas negative in TSIA medium,

urease negative and growth on cetrimide agar with production of pigments and fruity odour. Antibiotic susceptibility testing of isolates was assessed as per CLSI (2013) guidelines following modified Kirby-Bauer disc diffusion method. For differentiation of MDRPA isolates, 4 different antipseudomonal classes i.e. carbapenem (Imipenem), cephalosporin (Ceftazidime), fluoroquinolone (Ciprofloxacin) and aminoglycoside (Amikacin) were used.

Only those MDR isolates resistant to Ceftazidime were tested for metallo- β -lactamase enzyme by two methods. Combined disc test were done by placing two 10 μ g Imipenem discs 25mm apart on the surface of Mueller Hinton Agar plates inoculated with a bacterial suspension equivalent to 0.5 McFarland standard. One of the disc was loaded with 10 μ l of 0.5M EDTA to obtain desired concentration of 750 μ g. After 24 hours of incubation at 37°C, an increase of ≥ 7 mm in the zone diameter of EDTA containing Imipenem disc compared to Imipenem disc alone was considered to be a positive test for the presence of an MBL. In double disc synergy test, a 10 μ g Imipenem disc was placed 20mm centre to centre from a blank disc containing 10 μ l of 0.5M EDTA on the surface of Mueller Hinton Agar inoculated with test organism. After incubation at 37°C overnight, an increase of ≥ 7 mm in the zone diameter of blank disc containing EDTA compared to Imipenem disc was considered to be a positive test for the presence of an MBL.

AmpC β -lactamase production was screened by Cefoxitin disc diffusion test. Isolates showing inhibition zone diameter <18mm (screening positive) were further subjected to disc antagonism test for inducible AmpC enzyme and AmpC disc test for the detection of plasmid AmpC β lactamases.

In disc antagonism test, 0.5 McFarland suspension of test isolate was swabbed on MuellerHinton agar plate and Cefotaxime (30 μ g) and Cefoxitin (30 μ g) discs were placed 20 mm apart from centre to centre. After incubation at 37°C overnight, isolates showing blunting of the Cefotaxime zone of inhibition adjacent to the Cefoxitin disc were considered positive for inducible AmpC β -lactamase.

In AmpC disc test, a lawn culture of 0.5 McFarland suspensions of *Escherichia coli* ATCC 25922 was inoculated on surface of MuellerHinton agar plate. Sterile disc (6mm) was moistened with sterile saline

(20µl) and inoculated with several colonies of test organism. The inoculated disc was then placed beside a 30 µg Cefoxitin disc, almost touching it, on the inoculated plate. After overnight incubation at 37°C, positive test was interpreted as a flattening or indentation of the Cefoxitin inhibition zone in the vicinity of the test disc and negative test as absence of distortion.

RESULTS

Out of 756 samples investigated, significant growth was observed in 242 of them, among which 75 clinical isolates were identified as *P. aeruginosa* by standard microbiological procedures showing prevalence of 9.92% (Figure 1).

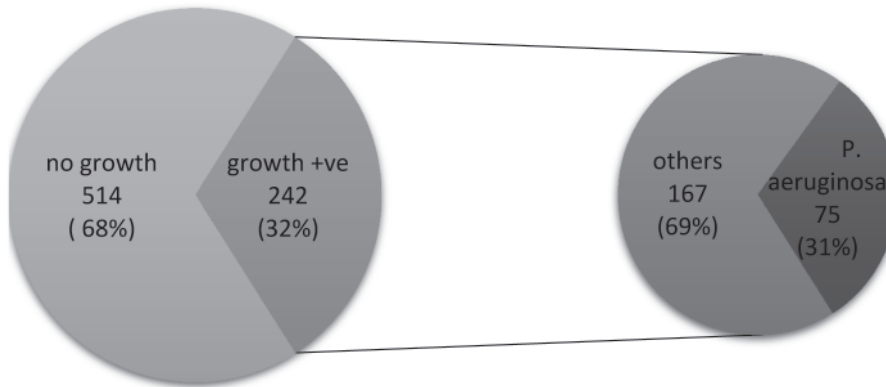


Figure 1: Prevalence of *P. aeruginosa* among clinical samples

Among different type of clinical samples analyzed, maximum number of *P. aeruginosa* was isolated from endo-tracheal (ET) secretions (69.3%) followed by

the category others (swabs, oral and nasal secretions) (12%), sputum (8%), urine (5.3%), blood (4%) and pus (1.3%) (Figure 2).

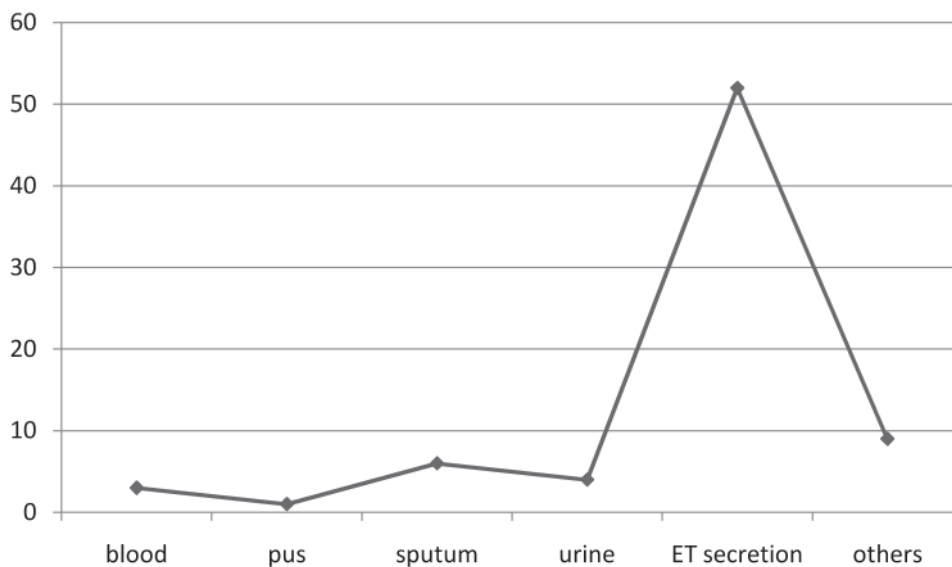


Figure 2: Distribution of *P. aeruginosa* among different specimens

Majority of the isolates were found to be resistant to most of the antibiotics tested. They showed highest resistance to Ceftazidime (88%) followed by Gentamicin (84%), Ofloxacin (82.7%) and Ciprofloxacin (78.7%).

Imipenem was found to be the most effective drug with susceptibility of 76% closely followed by Piperacillin-Tazobactam to which 74.7% of the organisms were susceptible (Figure 3).

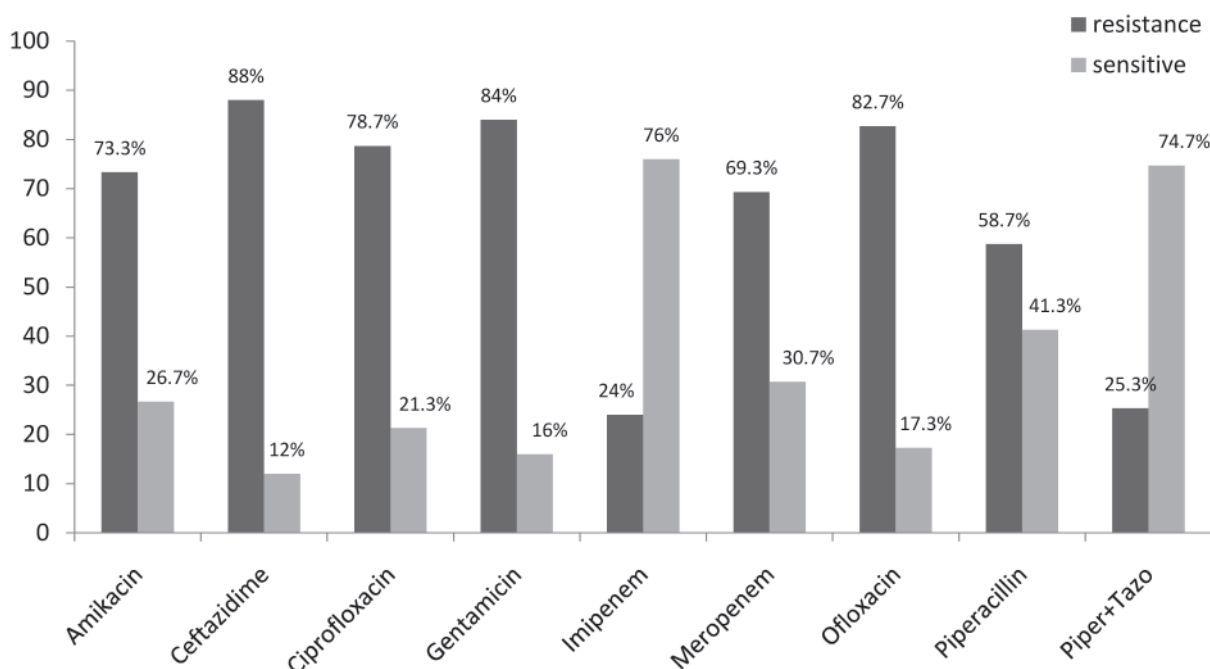


Figure 3: Antibiotic susceptibility pattern of *P. aeruginosa*

Out of 75 isolates, 70.6% of them were found to be multi-drug resistant *P. aeruginosa* (MDRPA). Out of 68 isolates from inpatient, 72.05% showed multi-drug resistance whereas in case of outpatient, out of 7 isolates, 4 of them

were MDR. Altogether out of 53 MDRPA, 92.45% were from inpatient whereas 7.54% were from outpatient. There was no significant association between the MDR occurrence and the type of patient ($p > 0.05$) (Table 1).

Table 1: Distribution of multi drug resistant *P. aeruginosa*

Type of strains	Type of patient		Total (%)	p-value
	Inpatient	Outpatient		
MDR	49(92.45%)	4 (7.54%)	53 (70.6%)	0.41
Non-MDR	19	3	22 (29.3%)	
Total	68	7	75 (100%)	

Among different types of clinical samples analyzed during the study, maximum no. of MDR isolates were observed in endotracheal (ET) secretion (73.58%) followed by the category others (9.43%), sputum (7.54%), and urine

(5.66%). Only 1.88% of the total MDR isolates were isolated from both blood and pus individually. The association between the MDR occurrence and specimen was found to be statistically insignificant ($p > 0.05$) (Table 2).

Table 2: Distribution of MDRPA among various samples

Type of Specimen	No. of <i>P. aeruginosa</i>	No. of MDR isolates	% of MDR isolates	p-value
Blood	3	1	1.88	0.55
Pus	1	1	1.88	
Sputum	6	4	7.54	
Urine	4	3	5.66	
ET secretion	52	39	73.58	
Others	9	5	9.43	
Total	75	53	100	

Ceftazidime resistant isolates were tested for MBL production. In this study, out of 53 MDR isolates, all were resistant to Ceftazidime. Altogether 11 (20.75%)

of them gave positive results for MBL by combined disc test and double disc synergy test (Figure 4).

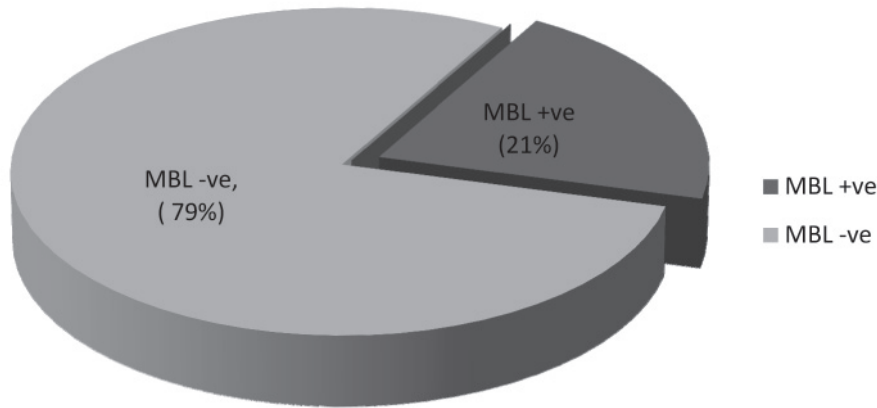


Figure 4: MBL production among MDR *P. aeruginosa*

Among two methods used for the detection of metallo-β-lactamase enzyme, double disc synergy test (DDST) showed only 7 out of 53 isolates (13.2%) to be positive for metallo-β-lactamase enzyme whereas combined

disc test (CDT) revealed 9 isolates (16.98%) to be positive for MBL. Five isolates gave positive result by both CDT and DDST methods whereas 2 isolates showed positive result by DDST only and 4 by CDT only (Table 3).

Table 3: Comparison of combined disc test and double disc synergy test

Combined disc test (CDT)	Double disc synergy test (DDST)	No. of isolates
+	-	4
-	+	2
+	+	5
-	-	42
Total		53

When screening for AmpC-β-lactamase producing organism was done by Cefoxitin disc diffusion test, out of 53 MDR isolates, 45 isolates were found to be

resistant which were further tested by Disc antagonism test for inducible AmpC and AmpC disc test for plasmid mediated AmpC (Figure 5).

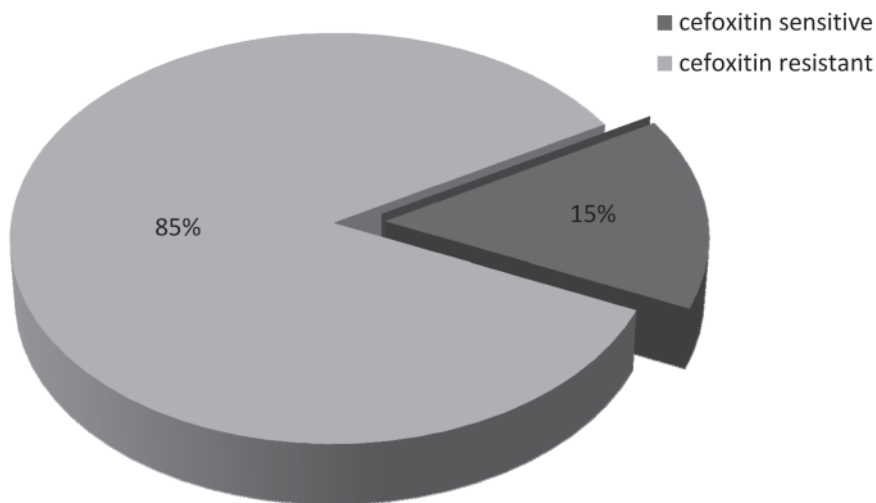


Figure 5: Cefoxitin susceptibility of the MDR isolates

Out of 45 positively screened MDR isolates, none of them were found to produce chromosomal (inducible) AmpC β -lactamase whereas 7(15.55%) were found to produce plasmid mediated (constitutive) AmpC β lactamase. Altogether out of 53 MDRPA, the prevalence of AmpC producing isolate was found to be 13.2%.

Among the 53 MDRPA strains which were screened phenotypically for various mechanisms of resistance, 20.75% were MBL producers, 13.2% AmpC producers, 3.77% produced both AmpC and MBL and 73.58% produced neither AmpC nor MBL (Figure 6).

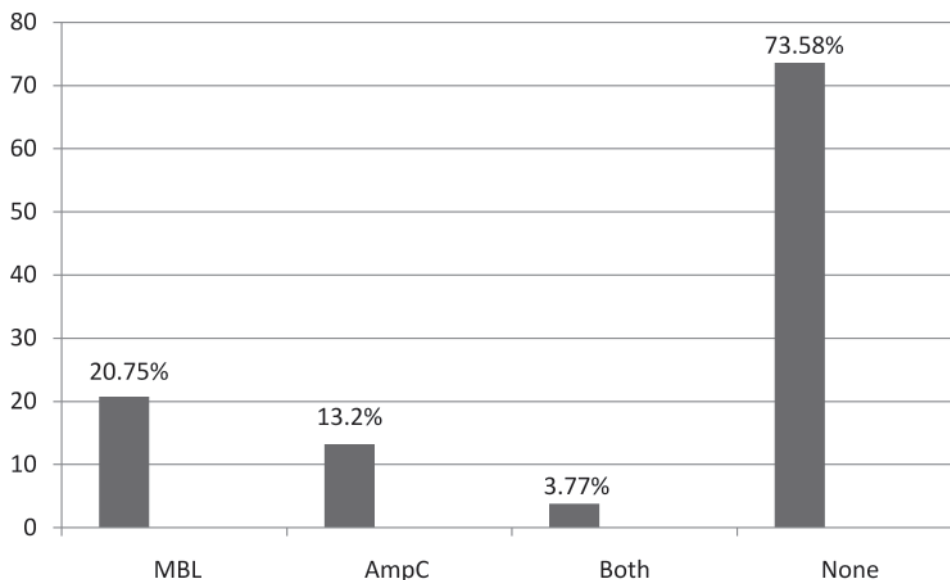


Figure 6: Various mechanisms of resistance among MDR isolates

DISCUSSION

A total of 756 clinical specimens from the patients of different age groups and wards were included in the study, out of which 75 (9.92%) of them were identified as *P. aeruginosa* by standard microbiological procedure. A recent study by Khan et al. (2014) reported similar prevalence of *P. aeruginosa* i.e. 6.67%. Another study by Mohanasoundaram (2011) showed the prevalence rate of *P. aeruginosa* to be 5%, 6.8% and 5% in three different years which is comparable with our studies. However a study done by Goel et al. (2009) reported a comparatively higher prevalence rate of *P. aeruginosa* (35%).

The distribution of specimens of *P. aeruginosa* may vary with each hospital as each hospital facility has a different environment associated with it. A study by Chander et al. (2013) showed the major source of *P. aeruginosa* pus/wound (27.6%) followed by sputum (24.1%), urine (20.7%), tracheal aspirate (10.35%), CVP catheter tube (3.45%), and 3.45% from each of BAL fluid, bile, catheter, and high vaginal swab. In another study by Basak et al. (2009), the clinical specimen following *P. aeruginosa* infections were most commonly from urinary

tract infection followed by wound infections. However a study done by Mohanasoundaram (2011) showed the maximum no. of isolates from pus and endotracheal secretions and Khan et al. (2014) in his study reported the isolation of maximum no. of *P. aeruginosa* from tracheal secretion and pus. In our study, maximum no. of isolates were obtained from endotracheal secretions (69.3%) followed by the category others (12%), sputum (8%), urine (5.3%), blood (4%) and pus (1.3%).

Increasing resistance to different anti-pseudomonal drugs particularly among hospital strains has been reported world-wide. In this study, the isolates showed highest resistance to Ceftazidime (88%) followed by Gentamicin (84%). The isolates resistant to Ciprofloxacin were 78.6%. These strains also showed resistance to carbapenems like Imipenem (24%) and Meropenem (69.3%), the precious weapon against *P. aeruginosa* infections (even against MDR isolates) which is an alarming sign. Imipenem was found to be the most effective drug with susceptibility of 76% followed by Piperacillin/Tazobactam (74.7%), Piperacillin (41.3%) and Meropenem (30.7%). Piperacillin alone tested showed a resistance rate of 58.7% in this study

whereas beta-lactam/beta-lactamase inhibitor drug Piperacillin/Tazobactam showed a lower resistance of 25.3% only, indicating beta-lactamase inhibitor markedly expands the spectrum of activity of beta-lactams, which makes the combination drug the preferred choice against *P. aeruginosa* infections. A recent study by Mohanasundaram (2011) with 193 *P. aeruginosa* showed 79% resistance to Gentamicin followed by Ceftriaxone (75%), Ciprofloxacin (73%), Ceftazidime (63%), Piperacillin (44%) and Amikacin (41.5%) which correlates with our study. They also reported resistance to Imipenem (3.7%) which is less compared to the present study. A study done by Landman et al. (2007) reported resistance of 24% to imipenem which exactly correlates with our study. In another study by Mohanasundaram (2011), the resistance rate to imipenem was 27% in 2009 and 16% in 2010 which is comparable with this study. Ceftriaxone and ceftazidime are commonest 3rd generation antibiotics in hospital protocols. In our study, the rate of aminoglycoside resistance was also found to be relatively high (Amikacin-73.3% and gentamicin 84%). This study shows that the clinical isolates of *P. aeruginosa* are becoming resistant to commonly used antibiotics and gaining more and more resistance to newer antibiotics.

A total of 53 out of 75 isolates were found to be multi-drug resistant in this study with the prevalence rate of MDRPA to be 70.6%. Moniri et al. (2005) reported a prevalence rate of 73.9% of MDRPA. A study by Mahmoud et al. (2012) and Ahmed et al. (2013) reported the prevalence of MDRPA to be 52% and 63.2% respectively which is also comparable to our study.

In this study, it was found that 92.45% of the total MDRPA was isolated from inpatients whereas only 7.54% belonged to outpatient. As observed in the study, the multidrug resistant strains of *P. aeruginosa* were mainly obtained from inpatients which might be due to increasing invasive procedures that are required for diagnosis and chemotherapy and predispose patients to acquire nosocomial infections with such pathogen. Ahmed et al. (2013) also mentioned the high prevalence of multi-drug resistant *P. aeruginosa* in inpatient than outpatient.

A study by Aggarwal et al. (2008) showed the major source of MDRPA were from sputum and tracheostomy specimen (28.57%), followed by pus (24.13%), urine (19.04%), cerebrospinal fluid and other

sterile body fluids (15.38%) and blood (7.14%). In a study by Shashikala et al. (2006), 27.6% of carbapenem resistant *P. aeruginosa* were obtained from surgical wounds and 20.7% were from endotracheal aspirate showing indwelling devices as major risk factors for the development of resistance. Present study revealed the major source of MDRPA to be endotracheal secretions (73.58%) followed by the category others (9.43%), sputum (7.54%), urine (5.66%) and 1.88% each from blood and pus.

The numerous β -lactamases are encoded either by the chromosomal genes or by the transferable genes which are located on the plasmids or the transposons. Among various mechanisms of resistance, AmpC and metallo- β -lactamase were detected in this study which is very effective in degrading the anti-pseudomonal agents these days. In 2002 from India, Navaneeth et al. reported MBL production in *P. aeruginosa* to be 12%. In the present study, we found 20.75% of MDRPA to be MBL producers. A recent study by Varaiya et al. (2008) showed 20.8% of MDRPA to be MBL producers. Ugargol (2012) in his study reported 18.8% of the isolates to be positive for MBL. Similarly, Navaneeth et al. (2002) and Willmann et al. (2013) respectively reported 18% and 15.9% of the MDRPA had the ability to produce MBL. Pandya (2011) showed MBL prevalence of 30%.

Since there is no standard guideline for detection of MBL production, different studies have reported to have used different methods. Two different phenotypic methods were used for the detection of metallo- β -lactamase in our study; Combined disc test (CDT) and Double disc synergy test (DDST). Combined disc test revealed 9 out of 53 i.e. 16.98% of the MDRPA isolates as MBL positive whereas Double disc synergy test showed only 7 out of 53 i.e. 13.2% as MBL producers. Five isolates gave positive result by both methods. In this study, both the combinations simultaneously detected 11 isolates i.e. 20.75% to be positive for MBL. In the evaluation of two selected MBL phenotypic assays, CDT was found to be more sensitive (16.98%), than DDST (13.2%). These results correlated with studies by Behera et al. (2008) (CDT- 88.8% and DDST-57.14%), and Pandya et al. (2011) (CDT-96.3%, DDST- 81.48%) both of which showed higher susceptibility of CDT than DDST although prevalence rate differs widely from our study. However, it differs from studies by Picao et al. at Brazil from January–December 2005 (CDT- 80%, DDST-82.6%) who showed DDST to be a

more sensitive method than CDT.

Out of 53 MDR isolates, 8 (15%) were found to be sensitive to cefoxitin whereas 45 of them i.e. 85% were resistant. A study done by Upadhyaya et al. (2010) reported 196 out of 202 isolates (97%) to be cefoxitin resistance which is comparable to our study. Hassan et al. (2011) in their study reported 57.33% (86/150) to be cefoxitin resistant. Similarly, another study by Behera et al. (2008) found 78.2% of the isolates to be cefoxitin resistant.

In our study, the overall prevalence of AmpC was found to be 13.2% which is comparable to study done by Basak et al. in 2009 (19.3%). Our study was also comparable with the reports from Aligarh by Shahid et al. in 2004 as 20% and from Kolkata by Arora et al. in 2005 as 17.3% and from Varanasi as 22%. Also, Ugargol (2012) in his study reported 12.8% of the isolates to be AmpC producer. However, Upadhyaya et al. (2010) reported a high prevalence of 59.4% of AmpC producing isolates out of which 7% were positive for inducible and 52.4% for non-inducible AmpC.

Co-production of AmpC and MBL was found in 3.77% of the MDRPA isolates in this study. Both of these isolates were from ICU and were resistant to imipenem. Singhal et al. (2005) showed the co-production of AmpC and MBL in 11% of the isolates. However, Upadhyaya et al. (2010) reported the co-production of AmpC and MBL in 46.6% of the isolates. Behera et al. (2008) showed the co-production of AmpC and MBL in 48.5% of the isolates. Both of these findings are comparatively much higher than our study. Production of multiple β -lactamases by *P. aeruginosa* has tremendous therapeutic consequences and there is a need for urgent action to control the spread of resistant strains. In our study, 39 MDRPA isolates were found to produce neither AmpC nor MBL. These strains may be harboring some other resistance mechanisms like biofilm formation or through virulence factors.

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