

Beta-lactamases in *P. aeruginosa*: A threat to clinical therapeutics.

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Abstract

Pseudomonas aeruginosa is the third most common pathogen responsible for nosocomial infections. The prevalence of multiple drug resistant *Pseudomonas aeruginosa* isolates harboring beta lactamases have been increasing. The present study is designed to determine the occurrence of various beta lactamases in *Pseudomonas aeruginosa*. A total of 237 clinical isolates of *P. aeruginosa* were tested for the presence of AmpC beta-lactamase, Extended Spectrum Beta-Lactamase (ESBL) and Metallo Beta-Lactamase (MBL) enzyme. Detection of ESBL was done by the combined disk diffusion method as per Clinical and Laboratory Standards Institute (CLSI) guidelines whereas MBL were detected by the Imipenem EDTA disk potentiation test and AmpC beta-lactamase was detected by disk antagonism test and modified three-dimensional method respectively. A total of 82 (34.60%) isolates were positive for AmpC beta-lactamase, 52 (21.94%) ESBL and 40 (16.87%) were positive for MBL. Co-production of AmpC with extended spectrum beta-lactamase and metallo beta-lactamase was reported in 20 (08.44%) and 24 (10.12%) isolates, respectively. All the three beta lactamases co-production was found to be in 7 (2.95%) isolates. The study emphasizes early detection of these multidrug resistant *P. aeruginosa* producing beta-lactamase enzymes of diverse mechanisms. Thus proper antibiotic policy and measures to restrict the indiscriminate use of antibiotics should be taken to minimize the emergence of these multiple beta-lactamase producing pathogens to avoid therapeutic failures and nosocomial outbreaks.

Keywords: Beta lactamase, *P. aeruginosa*, Amp C, MBL, ESBL.

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Introduction

Pseudomonas aeruginosa is a ubiquitous organism. It is found in environment and living sources like plants, animals and humans. *Pseudomonas aeruginosa* is also a major pathogen frequently implicated in Healthcare-Associated Infections (HAIs), particularly in critically ill or immunocompromised patients [1,2].

Nosocomial infections caused by this organism are often difficult to treat because of resistance to different antibiotics. Multidrug resistance in *P. aeruginosa* results from the bacterium's notable inherent antibiotic resistance, in addition to its ability to acquire and harbour diverse resistance determinants [3].

Acquired resistance is through the production of AmpC Beta Lactamases (AmpC), Extended Spectrum Beta Lactamases (ESBL) and Metallo Beta-Lactamases Enzymes (MBL). Resistance to β -lactam antibiotics is associated with production of ESBL which can hydrolyze oxymino β -lactams such as cefotaxime, ceftriaxone, ceftazidime and monobactams, however, without any effect on cephamycins, carbapenems and related compounds [4,5].

AmpC β -lactamases preferentially hydrolyze cephalosporins and cephamycins and resist inhibition by clavulanate, sulbactam and tazobactam. MBLs hydrolyze carbapenems and other beta-lactams. Resistance to carbapenems is of great concern as these are considered to be antibiotics of last resort to combat infections by multidrug-resistant bacteria [6].

The Multidrug Resistant (MDR) isolates that are present in the hospital environment pose not only therapeutic problems but also serious concerns for infection control management.

Material and Methods

The present study is a cross sectional study of a total of 237 consecutive non-repetitive isolates of *P. aeruginosa* obtained from 11,251 different clinical specimen, e.g. pus, urine, blood culture, respiratory tract, other samples like body fluid, drain fluid, etc. from hospitalized patients. The study was carried out in the Department from November 2013 to October 2015.

All the confirmed *P. aeruginosa* isolates were subjected to antimicrobial susceptibility testing by the Kirby-Bauer's

disc diffusion method as per the Clinical and Laboratory Standards Institute (CLSI) guidelines [7,8].

β -Lactamase Detection Tests

A. ESBL production by phenotypic confirmatory disk diffusion test [8]: Combined disk diffusion method was done using cefotaxime (30 μ g) and ceftazidime (30 μ g) disc alone and in combination with clavulanic acid disc (30/10 μ g). The test organism was inoculated on Muller Hinton Agar plate; discs were placed and incubated overnight at 37°C.

Interpretation: - Isolates showing zone of inhibition of ceftazidime plus clavulanic acid disc \geq 5 mm than those of ceftazidime disc alone was interpreted as ESBL producers (Colour plate Figure 1).

B. MBL production done by the disk potentiation test [9]: Two 10 μ g imipenem disks were placed on the plate, and appropriate amounts of 10 μ L of EDTA solution were added to one of them to obtain the desired concentration (750 μ g). The inhibition zones of the imipenem and imipenem-EDTA disks were compared after 16 to 18 h of incubation.

Interpretation: - An increase in zone size of \geq 7 mm around the Imipenem-EDTA disk as compared to the imipenem only disk was recorded to be MBL producers (Colour plate Figure 2).

C. AMPC β - Lactamase detection: Screening test was performed by cefoxitin (30 μ g) disk. Isolates that yielded a zone diameter less than 18 mm (screen positive) were further subjected to confirmatory testing by disc antagonism test and three dimensional tests.

1) Disk antagonism test [10]: Test isolate with a turbidity equivalent to that of 0.5 McFarland standards was spread over a Mueller Hinton agar plate. Cefotaxime (30 μ g) and cefoxitin (30 μ g) disks were placed 20 mm apart from centre to centre. Isolates showing blunting of cefotaxime zone of inhibition adjacent to the cefoxitin disc were screened as positive for AmpC betalactamase (Colour plate Figure 3).



Figure 1. ESBL production by *P. aeruginosa*

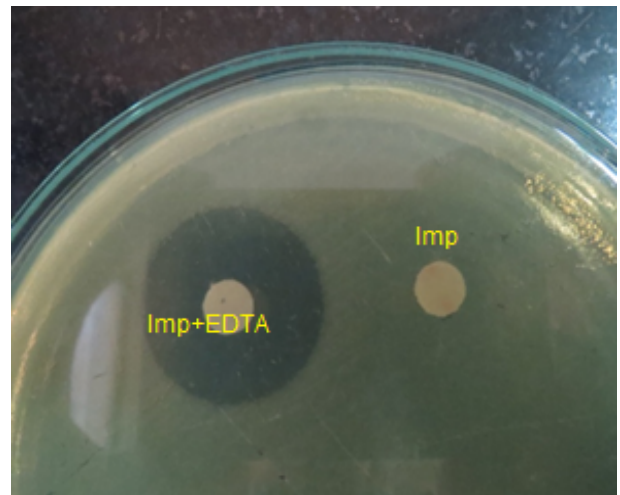


Figure 2. MBL production by *P. aeruginosa*



Figure 3. Inducible AmpC β -lactamase production in *P. aeruginosa*

2) Modified three dimensional test for AmpC [4]: Fresh overnight growth from Mueller Hinton agar was transferred to pre-weighed sterile micro centrifuge tube. Technique was standardized to obtain 10-15 mg of bacterial weight for each sample. Growth was suspended in peptone water and was pelleted by centrifugation at 3000 rpm for 5 min.

Crude enzyme extract was prepared by repeated freeze thawing (five rounds) Lawn culture of *e. coli* ATCC 25922 was prepared on Mueller Hinton agar plates and cefoxitin (30 μ g) discs were placed on plate. Linear slits (3 cm) were cut using a sterile surgical blade 3 mm away from cefoxitin disc. Small circular wells were made on slits at 5 mm distance, inside the outer edge of slit, by stabbing with sterile Pasteur pipette on agar surface. The wells could easily be loaded with enzyme extract in 10 μ l increments until well was filled to the top. Approximately 30-40 μ l of extract was loaded in wells. The plates were kept upright for 5-10 min until the solution dried and were then incubated at 37°C overnight. Enhanced growth of surface organism at point where the slit inserted zone of inhibition of cefoxitin was considered a positive three dimensional test and was interpreted as evidence of AmpC beta-lactamases.

Interpretation: Isolates showing clear distortion of zone of inhibition of cefoxitin were taken as AmpC producers.

Isolates with no distortion were recorded as non AmpC producers. Isolates showing minimal distortion were considered as indeterminate strains.

Results

Antibiotic sensitivity testing of *P. aeruginosa*, in present study, revealed maximum resistance to (63.71%) piperacillin, followed by ciprofloxacin (62.87%), tobramycin (53.16%), ceftazidime (51.05%), cefepime (49.79%), aztreonam (49.37%), gentamicin (47.68%), norfloxacin (47.68%), piperacillin/tazobactam (42.19%) and amikacin (37.97%). Low resistance were seen to imipenem (21.94%), polymyxin B (05.49%) and no resistance to colistin (Table 1).

As high resistances were seen to beta-lactam antibiotics we tested for beta-lactamase production in *P. aeruginosa*. We found multiple beta-lactamase production in *Pseudomonas* spp. AmpC to be most common β -lactamases followed by ESBL and MBL respectively. Multiple β -lactamase production was seen in 21.52% isolates, i.e., production of 2 or 3 β -lactamases simultaneously (Table 2).

Out of 237 *P. aeruginosa* isolates tested, ceftoxitin resistance was evident in 218 (91.98%) isolates while 82 (34.60%) isolates were confirmed to be AmpC producer. Among the test isolates, 68 (28.69%) were detected as inducible AmpC producers while 14 (5.91%) of the isolates were confirmed to be non-inducible (Table 3).

We found co-expression of various β -lactamases in multidrug resistant clinical isolates of *Pseudomonas aeruginosa*. In present study 10.12% isolates showed co-existence of AmpC+MBL, 8.44% were having ESBL+AmpC and 2.95% isolates were having co-existence of all three ESBL+AmpC+MBL. Expression of AmpC and MBL simultaneously was found to be the most common mechanism of drug resistance in present study (Table 4).

Furthermore, all beta-lactamase producing isolates of *Pseudomonas aeruginosa* were resistant to 3 or more antibiotic classes indicating multidrug resistance.

Table 1. Antibiotic resistance pattern of *Pseudomonas aeruginosa*

Antibiotic Disc	<i>P. aeruginosa</i> (%)
Piperacillin (100 μ g)	151 (63.71)
Piperacillin/Tazobactam (100/10 μ g)	100 (42.19)
Ceftazidime (30 μ g)	121 (51.05)
Cefepime (30 μ g)	118 (49.79)
Aztreonam (30 μ g)	117 (49.37)
Imipenem (10 μ g)	52 (21.94)
Colistin (10 μ g)	00 (00.00)
Polymyxin B (300 units)	13 (05.49)
Gentamicin (10 μ g)	113 (47.68)
Tobramycin (10 μ g)	126 (53.16)
Amikacin (30 μ g)	90 (37.97)
Ciprofloxacin (5 μ g)	149 (62.87)
Norfloxacin (10 μ g) (n=44)	21 (47.72)

Discussion

β -Lactamases in *P. aeruginosa*

There are variable reports of different β -lactamases production in *P. aeruginosa* species. In present study, AmpC β -lactamase was found to be most common β -lactamase in *Pseudomonas aeruginosa* isolates. Rodrigues et al. [11], Kumar et al. [6], Altun et al. [12] have also found AmpC to be most common β -lactamase. But Nagdeo et al. [13], Goel et al. [14] reported ESBL and MBL to be most common β -lactamase among *Pseudomonas aeruginosa* in their study.

The percentage of AmpC β -lactamases in present study is similar to Kumar et al. [12] (32.7%), Nagdeo et al. [13] (30.88%).

Inducible AmpC (28.69%) (Table 4) in present study is similar to findings of Rodrigues et al. [11] (26.5%), Bhattacharjee et al. [15] (22%) and Kumar et al. [6] (27.7%). Non-inducible AmpC (5.91%) in present study is similar to findings of Kumar et al. [6] (5%) butin contrasts with Rodrigues et al. [11] (32.4%) and Upadhaya et al. [16] (52.4%).

The ESBL production in present study (21.94%) is comparable with Aggarwal et al. [17] and Peshattiwar et al. [18] (22.22%) and Sheikh et al. 2015 (25.13%) [19]. These observations suggest that the ESBLs which were generally widespread among members of Enterobacteriaceae are also increasingly found in *P. aeruginosa*.

MBL production in present study (16.87%) is comparable with Attal et al. [20] (11.4%) and Bashir et al. [21] (11.66%). In present study, multiple β -lactamase production was present in 21.52% isolates which is comparable with Rodrigues et al. [11] (22.1%), Kumar et al. [6] (23.76%) and Goel et al. [14] (23.08%). Nagdeo et al. [13] (28.57%) and Upadhaya et al. [16] (29.7%) showed slightly higher percentage of multiple β -lactamase production as compared to present study. The differences seen in the percentage and the type of β lactamases is probably due to the local hospital antibiotic policy resulting in drug pressure and development of resistance by different enzyme expression.

Co-Expression of β -Lactamases in *Pseudomonas aeruginosa*

The incidence of coexistence of different β -lactamases in present study is comparable with Nagdeo et al. [13], Goel et al. [14]. Co expression of MBL and AmpC in present study was found to be highest among all co expressions. Similarly Upadhayay et al. [16], Kumar et al. [6], Nagdeo et al. [13] and Salimi et al. [22] had got MBL+AmpC coexistence higher than other combinations of beta lactamases. Upadhayay et al. [16] (46.6%) and Salimi et al. [22] (81%) reported higher percentage of MBL+AmpC co-existence. Incidence of co-expression of AmpC+ESBL was lower in our study as compared to incidence from Rodrigues et al. [11] (22.1%) and Easwaran et al. [23] (68.71%).

Table 2. Different β -lactamases production in *Pseudomonas aeruginosa* species

Beta Lactamases	Single Enzyme (%)	Total Number (%) n=237
AmpC	31 (13.08%)	82 (34.60)
ESBL	25 (10.55%)	52 (21.94)
MBL	9 (3.80%)	40 (16.87)
Multiple β -lactamase	–	51 (21.52)

Table 3. Total number of inducible as well as non-inducible AmpC β -lactamase producers in *P. aeruginosa*

Total number of <i>P. aeruginosa</i> isolates	Screening Positive (Cefoxitin resistance)	Confirmatory positive	
		Disk antagonism test (inducible AmpC producers)	Modified 3 dimensional test (inducible and non-inducible AmpC producers)
237	218 (91.98%)	68 (28.69%)	82 (34.60%)

Table 4. Co expression of various β -lactamases in *P. aeruginosa*

β -lactamases	Number (%)
AmpC+MBL	24 (10.12)
AmpC+ESBL	20 (08.44)
ESBL+MBL+AmpC	7 (2.95)

Multiple β -lactamase producing *P. aeruginosa* can cause major therapeutic failure and pose a significant clinical challenge if they remain undetected. Since these organisms also carry other drug-resistant genes the only viable treatment option remains the potentially toxic Polymyxin B and Colistin [24].

The isolates producing different β -lactamase in present study were found to be multidrug resistant, i.e., all AmpC, ESBL, MBL producing isolates of *P. aeruginosa* were resistant to ≥ 3 antibiotics. This correlates with the findings of De et al. [25], Peshattiwar et al. [18], Bashir et al. [20] and Salami et al. [22] which showed all MBL isolates to be multidrug resistant. Similar to present study, Upadhaya et al. [16] also reported all AmpC producers to be multidrug resistant. Glupczynski et al. [26] reported 100% ESBL producers to be multi-drug resistant similar to present study.

The finding of multidrug resistance among β -lactamase producing *P. aeruginosa* could be due to co-existence of genes encoding drug resistance to these antibiotics on the plasmids, transposons and chromosomes carrying these beta-lactamases.

Conclusion

The present study emphasizes production of multiple β -lactamase enzymes by *P. aeruginosa* leading to multidrug resistance. Early detection of these β -lactamase producing isolates in a routine laboratory could help prevent treatment failure, as often the isolates producing this enzyme show a susceptible phenotype in routine susceptibility testing.

Furthermore, strict implications of antibiotic policies and measures to limit the indiscriminate use of antibiotics in hospital environment should be undertaken to minimize the emergence of this multiple β -lactamase producing organism. Thus microbiologists in India have a very important role in prevention of spread of this dreaded multidrug resistant pathogen across the world.

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