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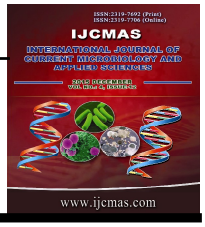

Original Research Article

Prevalence of Antimicrobial Resistance in Clinical Isolates of *Pseudomonas aeruginosa* in a Tertiary Care Hospital, Puducherry, India

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Extended spectrum beta-lactamase, AmpC beta-lactamase, Metallo beta-lactamase

A B S T R A C T

Pseudomonas aeruginosa isolates are well known cause of outbreaks of various nosocomial infection. These organism are difficult to treat as they exhibit varying degrees of innate and acquired resistance mechanism. This study was undertaken to analyse various acquired resistance mechanism like Extended spectrum β-lactamase (ESBL), Metallo β-lactamase (MBL) and inducible AmpC beta (β)-lactamase, enzymes in clinical isolates of *Pseudomonas aeruginosa*. The study included a total of 203 consecutive non-duplicate isolates of *Pseudomonas aeruginosa* obtained from clinical specimens like pus (103), urine (50) and sputum (50). Phenotypic detection of Extended spectrum beta-lactamase (ESBL), Metallo β-lactamase (MBL) and AmpC beta-lactamase (AmpC β-lactamase)of *Pseudomonas aeruginosa* were done by Phenotypic confirmatory disc diffusion test, Disk potentiation test and Disk antagonism test respectively. Of 203 isolates, 49(47.6%), 52(50.5%) and 21(20.4%) were Extended spectrum beta-lactamase(ESBL), Metallo β-lactamase and AmpC β-lactamase producers respectively..In conclusion, our study has shown high incidence of ESBL and MBL producing *Pseudomonas aeruginosa*. Multi-drug resistance were found to be 52 (25.6%) Henceforth, strict antibiotic policies and measures to limit the indiscriminative use of cephalosporins and carbapenems in the hospital environment should be undertaken to minimize the emergence of multiple β-lactamase.

Introduction

Pseudomonas aeruginosa isolates are responsible for outbreaks of nosocomial infection in different parts of the world. These isolates have also been responsible for serious infections such as septicaemia and pneumonia. *Pseudomonas aeruginosa* infections are difficult to treat as they exhibit varying degrees of innate and acquired resistance mechanism, when there is a rise of resistance against multiple antimicrobial drugs which leads to high morbidity and mortality (Zavascki AP *et al*., 2010).

Restricted permeability and efflux are common components of the resistance phenotype for β -lactams, aminoglycosides and quinolones which are essentially

fundamental properties of the organism. The innate antibiotic resistance of *Pseudomonas aeruginosa* results from the restricted permeability of the cell wall and is enhanced by the activity of efflux systems

Resistance to β -lactams involves several mechanisms, the most prevalent mechanism of β -lactam resistance is the production of β -lactamases, followed by permeability alterations, extrusion by efflux pumps, and to a lesser extent PBP alterations. Acquired resistance mechanism includes plasmid mediated AmpC beta (β)-lactamase, extended spectrum β-lactamase and metallo β-lactamase (MBL) enzymes (Manchanda V *et al*., 2010).

Materials and Methods

A total of 203 consecutive non-duplicate isolates of *Pseudomonas aeruginosa* obtained from clinical specimens like pus (103), urine (50) and sputum (50) were included in this study. All isolates will be subjected to routine antibiotic susceptibility testing by Kirby Bauer method according to CLSI guidelines (CLSI, 2014).

Antimicrobial susceptibility was performed by the Kirby-Baeur disk diffusion method for various antibiotics, namely: Amikacin (30μg), Gentamicin (10μg), Netilmicin (30μg), Tobramycin(30μg), Ciprofloxacin (5μg), Norfloxacin (10μg), Ceftazidime (30μg), Imipenem (10μg), Piperacillin/ tazobactam (100μg/10μg).

Detection of Antibiotic Resistance in *Pseudomonas aeruginosa*

Phenotypic detection of Extended spectrum beta-lactamase (ESBL), AmpC beta-lactamase and Metallo β-lactamase (MBL) of *Pseudomonas aeruginosa* was done by the following methods;.

Detection of Extended Spectrum Beta-Lactamase (ESBL)

All isolates were tested for their susceptibility to the 3rd generation cephalosporins (3GCs)s ceftazidime (30 μg/disk), cefotaxime (30μg/disk) and ceftriaxone (30 μg/disk) by using the standard disc diffusion method as recommended by CLSI [3]. Isolates which were resistant to at least one of the 3GCs were selected for the study and were processed for ESBLs production. If a zone diameter of ≤ 22 mm for ceftazidime, ≤ 27 mm for cefotaxime and ≤ 25 mm for ceftriaxone were recorded, the isolate was considered to be "suspicious for ESBL production".

All isolates were confirmed for ESBL production using Phenotypic confirmatory disc diffusion test /Combined disk diffusion method (CLSI, 2014).

Phenotypic Confirmatory Disc Diffusion Test (PCDDT)/ Combined Disk Diffusion Method

Ceftazidime and cefotaxime discs (30μg) alone and in combination with clavulanic acid (30/10μg) were applied onto plate of Mueller Hinton agar, inoculated with the test isolates. Diameter of zone of inhibition was measured after overnight incubation at 37ºC. An increase of ≥ 5 mm diameter in a zone of inhibition of the combination discs in comparison to the cefotaxime or ceftazidime was considered to be a marker for ESBLs producing isolates (Fig.1).

Detection of Metallo β-Lactamase

The carbapenem resistant Pseudomonas spp were further screened for MBL production by Disk potentiation test (Yong D *al*., 2002).

Disk Potentiation Test

Metallo β-lactamase production was detected by Imipenem-EDTA disk test. Two 10 μg imipenem disks were placed on the plate, to one of the disk 10 μl of 50mM zinc sulphate was added after drying, 5μl of 0.5M EDTA solution was then dispensed(930 μg per disc). The inhibition zones of imipenem and imipenem-EDTA disks were compared after 16 to18 hours of incubation in air at 35˚C. If the increase in inhibition zone with imipenem and EDTA disk was ≥ 7 mm, then the imipenem disk alone was considered to be the MBL producer(Fig.2) (Yong D *al*., 2002).

Detection of AmpC β -Lactamase

Screening for AmpC β-lactamase production was performed by Cefoxitin disk test. Isolates that yielded a zone diameter less than 18 mm (screen positive) were further subjected to disk antagonism test for detection of inducible AmpC β-lactamase in all the isolates of *Pseudomonas aeruginosa.*

Disk Antagonism Test

A test isolate (with a turbidity equivalent to that of 0.5 McFarland standards) was spread over a Mueller Hinton agar (Hi-Media) plate. Cefotaxime (30μg) and cefoxitin (30μg) (Hi-Media Mumbai) disks were placed 20 mm apart from centre to centre. Isolates showing blunting of the cefotaxime zone of inhibition adjacent to the cefoxitin disk were screened as positive for AmpC β-lactamase (Fig.3) (Upadhyay *et al*., 2010).

Results and Discussion

A total of 203 strains of *Pseudomonas aeruginosa* were studied for antimicrobial susceptibility patterns.

Of which, *Pseudomonas aeruginosa* showed maximum resistance to Gentamicin (35.5%), followed by Ceftazidime (32.5%), Ciprofloxacin(30%) and least resistant to Imipenem(8.9%) as shown in Table 1. Resistance to ≥ 3 different classes of antibiotics tested were taken as Multi-drug resistance (MDR) and 52(25.6%) of *Pseudomonas aeruginosa* isolates were found to be MDR strains

Out of 203 *Pseudomonas aeruginosa* isolates tested, isolates showing resistance to cefoxitin were confirmed for AmpC β-lactamase producers by Disc antagonism were 21(20.4%). The Extended spectrum beta-lactamase (ESBL) detection by combined disk diffusion method and Metallo β-lactamase production by Imipenem-EDTA disk test showed 49(47.6%) and 52(50.5%) respectively as shown in Table 2.

In table 3, Co- existence of ESBL and MBL were shown in 22(10.8%) isolates. But combination of ESBL with AmpC and MBL along with AmpC were shown in 8(3.9%) and 7(3.4%) only. All three beta-lactamase production were also noted in 4(1.9%) of 203 isolates of *Pseudomonas aeruginosa*.

Pseudomonas aeruginosa is known for intrinsic resistance to various antibiotics because of several factors like low permeability of outer-membrane, the constitutive expression of various efflux pumps, and the production of antibiotic-inactivating enzymes. By inappropriate and widespread use of antibiotics, bacteria adopt to develop newer resistant mechanisms to escape the action of the antibiotics.

Routine antibiotic susceptibility testing for *Pseudomonas aeruginosa* in our study we observed maximum resistance to aminoglycosides like Gentamicin(35.5%) and ceftazidime(32.5%) followed by ciprofloxacin(30%) and least resistant to

Imipenem(8.9%) More than 80% susceptibility were shown for Imipenem and Piperacillin –Tazobactum. There are several studies showing resistance pattern of ceftazidime above 60% (Kaur DC *et al* ., 2013; Zafer MM *et al* ., 2014), ciprofloxacin from 48-59% (Chaudhari VL *et al* ., 2013; Amutha R *et al* ., 2009), much higher than our study, whereas another study (Joseph NM *et al*., 2013) showed that significant reduction in resistant rate of ceftazidime from 50% to 33% and of ciprofloxacin from 49% to 33% comparable to our study . Studies showing better antipseudomonal activity with amikacin and imipenem similar to our study. (Chaudhari VL *et al* ., 2013; Amutha R *et al* ., 2009; Joseph NM *et al*., 2013; Senthamarai S *et al*., 2014).

In recent years, *Pseudomonas aeruginosa* infections are difficult to treat due to emergence of newer β-lactamases such as Extended Spectum β-lactamases (ESBL), AmpCβ- lactamases and Carbapenemases. Major mechanisms causing resistance to the β-lactam antibiotics in *Pseudomonas aeruginosa* are the production of β-lactamases, reduced outer membrane permeability and altered affinity of target Penicillin binding proteins (Washington CW, 2006).

The β-lactamases inactivate β-lactam antibiotics by cleaving the structural β-lactam ring. ESBL-producing bacteria are frequently resistant to many other classes of antibiotics, including aminoglycosides and fluoroquinolones. This is due to the coexistence of genes encoding drug resistance to other antibiotics on the plasmids which encode ESBL (Nathisuwan S, 2001).

Combined double disc synergy test showed 47.6% of 203 total *Pseudomonas aeruginosa* isolates confirmed positive for ESBL production in our study. Of these, maximum ESBL production were seen in isolates from Pus(59.6%) followed by Urine(23.1%) and Sputum(17.3%).This finding was supported by results of previous studies which showed ESBL production around 40% ,42.3% (Senthamarai S *et al*., 2014 ; Goel V *et al* ., 2013; Silpi Basak *et al* ., 2012). In another study (Aggarwal *et al*., 2008) the percentage of of ESBL production in sputum (28.57%) and urine (19.04%) were correlating with our study except that of pus, where we recorded higher percentage. In present study, of 66 ceftazidime resistant strain, only 48 (72.7%) were confirmed positive for ESBL .These resistant strains may possess other enzyme mediating resistance such as MBL production, AmpC beta lactamase and /other resistance mechanisms such as membrane permeability and efflux mechanisms .

Carbapenems are now being considered the drug of choice for treatment of infections in extended spectrum β lactamase (ESBL) producing Gram-negative bacteria (Mendiratta DK *et al*., 2005). However, carbapenem resistance due to decreased outermembrane permeability, increased efflux systems, alteration of penicillin binding proteins and carbapenem hydrolyzing enzymes-carbapenemase has been observed frequently in non fermenting bacilli like *Pseudomonas aeruginosa* and *Acinetobacter spp*. (Gladstone P *et al*., 2005). Emergence and spread of MBL‑mediated resistance is of serious concern as they would restrict the therapeutic options(Nordmann P *et al*.,2002). In the current study, 50.5% of 203 total *Pseudomonas aeruginosa* were positive for Metallo β-lactamase production by Imipenem-EDTA disc test, with maximum incidence in pus (63.3%), followed by urine (26.5%) & sputum(10.2%).Our finding agreed with study by Goel V *et al*.,2013, in which 53.85% of *Pseudomonas aeruginosa*

were MBL Producers. Another study (Kaur DC *et al*., 2013) recorded highest prevalence of Metallo-β-Lactamases in Pus (41.66%) followed by Urine (33.33%) and sputum (20%) in concordance with our study.

In the present study, 20.4% of total 203 *Pseudomonas aeruginosa* were screened positive for inducible AmpC β-lactamase by Disc antagonism test .This result agreed with several other studies in India% (Arora S *et al*., 2005 ; Shahid S *et al* ., 2004;Bhattacharjee *et al* ., 2008) which showed similar results (17.3%,20%,, 22%) for AmpC production.

Table.1 Antibiotic Susceptibility Pattern of *Pseudomonas Aeruginosa*.(n=203)

Antibiotics	**Sensitive(%)**	**Intermediate(%)**	**Resistant(%)**
Amikacin	146(71.9%)	14(6.89%)	43(21.2%)
Gentamicin	106(52.2%)	25(12.3%)	72(35.5%)
Tobramycin	157(77.3%)	6(2.95%)	40(19.7%)
Ciprofloxacin	132(65%)	10(4.9%)	61(30%)
Ceftazidime	130(64%)	7(3.4%)	66(32.5%)
Piperacillin – tazobactum	164(80.8%)	15(7.4%)	24(11.8%)
Imipenem	179(88.2%)	6(2.95%)	18(8.9%)

Table.2 Distribution of β-Lactamase in Clinical Isolates of *Pseudomonas Aeruginosa* (n=203)

Sample	**ESBL(%)**	**MBL(%)**	**AmpC(%)**
Pus(103)	31(59.6%)	31(63.3%)	10(47.6%)
Urine(50)	12(23.1%)	13(26.5%)	8(38.1%)
Sputum(50)	9(17.3%)	5(10.2%)	3(14.3%)
Total (203)	**52(50.5%)**	**49(47.6%)**	**21(20.4%)**

Table.3 Co- existence of Different β-Lactamase Resistance Mechanism in *Pseudomonas aeruginosa* (n = 203)

Multiple resistance	**Number**	**%**
ESBL+MBL	22	10.8%
ESBL+AmpC	8	3.9%
MBL+AmpC	7	3.4%
ESBL+AmpC+MBL	4	1.9%

Figure.1 ESBL Production in *Pseudomonas aeruginosa* is Shown above by Increased zone of Inhibition in Ceftazidime + Clavulanic Acid(CAC) Compared to Ceftazidime Discs (CAZ)alone by Combined Disc Diffusion Test

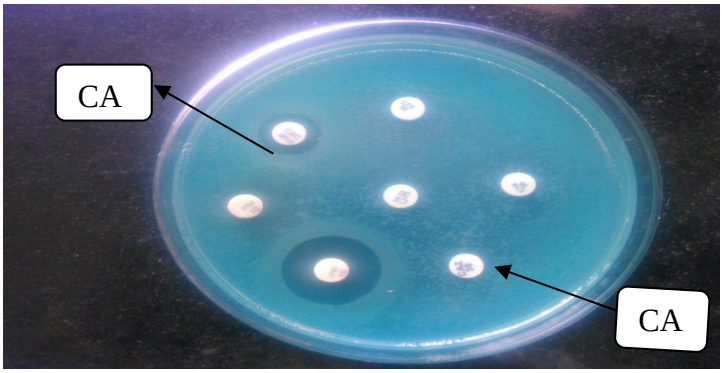


Figure.2 MBL Production in *Pseudomonas aeruginosa* is Observed by Enhanced Zone Size in Imipenem + EDTA Combination than Imipenem (IPM) alone by **Disc Potentiation Test**

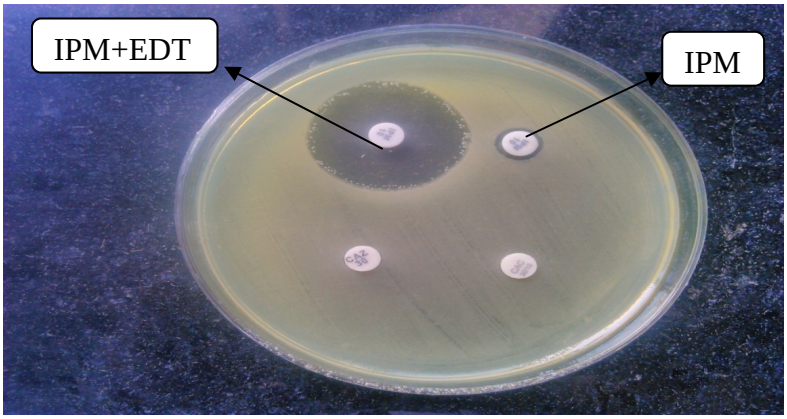
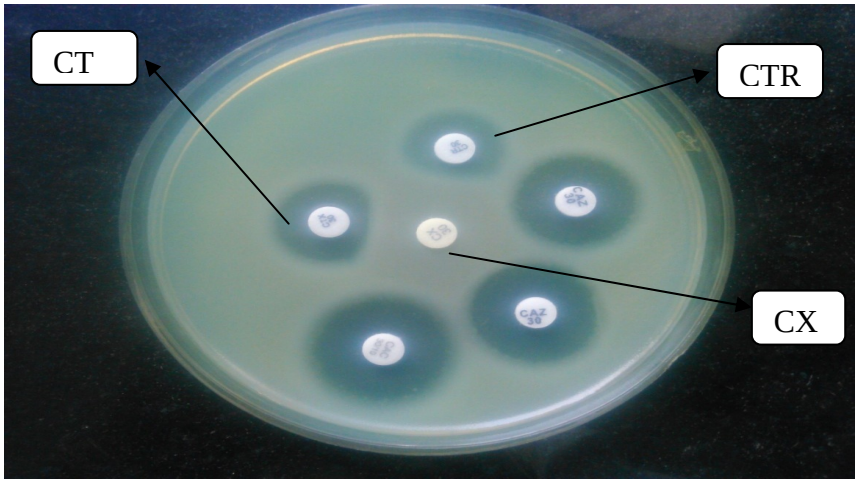


Figure.3 Screening of Inducible AmpC Beta Lactamase in *Pseudomonas aeruginosa* by **Disc Antagonism Test** is Showen by Blunting of Cefotaxime(CTX) and Ceftriaxone(CTR) Zone Adjacent to Cefoxitin(CX) Disc



In the current study, co-existence of different β-lactamases mediated resistance mechanism in *Pseudomonas aeruginosa* were analysed..Among the test isolates, we reported 10.8% co-existence for ESBL and MBL producers. Of 203 isolates of *Pseudomonas aeruginosa* ,3.9% had both ESBL and AmpC production, co-existence of MBL along with AmpC were reported in 3.4% .Study (Upadhyay *et al* ., 2010) reported 3.3% co-existence of AmpC along with extended spectrum beta-lactamase ,in concordant to our study . Another study (Silpi Basak *et al.*,2012*)* showed 27.2% of co-existence of ESBL and AmpC β-lactamases higher than the present study. Production of multiple β-lactamases by P. aeruginosa poses a great threat to treatment of multidrug resistant strain in near future.

In current study, Multi-drug resistant (MDR) strains were found to be 52 (25.6%). Study from Nepal (Anil C *et al*.,2013)reported similar incidence MDR (20.69%) *Pseudomonas aeruginosa*. There are studies showing decrease in rate of MDR *Pseudomonas aeruginosa* from 37.9% in 2007 to 23.7% in 2012(Chaudhari VL *et al*., 2013; Joseph NM *et al* ., 2013) . Certain other studies have even reported higher percentage (41.35%,91.6%) (Senthamarai S *et al*., 2014; Paranjothi S *et al* ., 2010) of MDR *Pseudomonas aeruginosa*.

In conclusion, current study have recorded quite highest prevalence (>45%) of ESBL and MBL producing *Pseudomonas aeruginosa* isolated from clinical samples and out of 203 isolates of *Pseudomonas aeruginosa* 25% were MDR strains.However, in our study we found that most of the strains were sensitive to Carbapenems, Piperacillin and Gentamicin. To minimize the emergence of multiple β-lactamase producing isolates in the laboratory, phenotypic detection of resistance pattern should be carried out along with routine susceptibility testing. Failure to detect these enzymes producing strains has contributed to their uncontrolled spread of resistant strain and therapeutic failure. In such cases treatment of severe infections requires combination therapy to combat multidrug resistant strains. Hence, strict antibiotic policies and measures to limit the indiscriminative use of cephalosporins and carbapenems in the hospital environment should be undertaken to minimize the emergence of this multiple β-lactamase.

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