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ORIGINAL ARTICLE

Inducible AmpC Beta-Lactamase Producing Pseudomonas Aeruginosa Isolated In A Rural Hospital Of Central India

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ABSTRACT

Pseudomonas aeruginosa, one of the most common pathogen causing nosocomial infections, shows increasing resistance to β -lactam antibiotics especially by producing AmpC β -lactamase. Hence, this study was undertaken to determine the prevalence of inducible AmpC β -lactamases producing *Pseudomonas aeruginosa* in our hospital and their antibiotic susceptibility pattern to newer antipseudomonal antibiotics.

Consecutive 244 *Pseudomonas aeruginosa* isolates were studied. Isolates showing blunting of ceftazidime zone of inhibition adjacent to ceftoxitin disc were considered as screen positive and were selected for confirmation of inducible AmpC β -lactamases producing by modified three dimensional test and AmpC disc test. In vitro susceptibility pattern of antipseudomonal antibiotics were done by Kirby Bauer disc diffusion method.

Out of 244 *Pseudomonas aeruginosa* isolates 47 (19.3%) were screen positive, which were confirmed by modified three dimensional test and AmpC disc test. The highest sensitivity pattern observed was Imipenem, Amikacin and Ciprofloxacin.

We conclude that to avoid misuse of antibiotics and to start proper antibiotics to hospitalized patients, tests for AmpC β -lactamases should be done in Clinical Microbiology laboratories.

Key Words: *Pseudomonas aeruginosa*, Inducible AmpC β -lactamase, AmpC disc test.

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The commonest mechanism of β -lactam antibiotic resistance in Gram negative bacteria is predominantly due to the production of β -lactamases that cleave the structural β -lactam ring. According to molecular structure classification by Ambler 1980 the major classes of β -lactamases are A, C, D [1].

Pseudomonas aeruginosa can produce all major classes of β -lactamases (A, C, D) and also metallo β -lactamases i.e. class B. Persistent exposure of *Pseudomonas aeruginosa* to β -lactam antibiotics leads to mutation and over production of AmpC or class C β -lactamases. These are cephalosporinases which were classified by Ambler in 1980 [1] as molecular class C and according to Bush et al [2] were classified as

Introduction

Pseudomonas aeruginosa is one of the most common pathogen causing nosocomial infection. It is intrinsically resistant to many antibiotics including newer β -lactam antibiotics or can develop resistance during treatment leading to high mortality and morbidity.

Group I. AmpC β -lactamases confer resistance to oxyiminocephalosporins, 7 α methoxycephalosporins (cefoxitin and cefotetan).

AmpC β -lactamases which are chromosomally mediated have been described in *Acinetobacter sp*, *Morganella morganii*, *Citrobacter freundii*, *Enterobacter sp*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Serratia marcescens*, *E.coli*, *Hafnia alvei* etc. In most of these bacteria AmpC is inducible via a system involving ampD, ampG, ampR and intermediates in peptidoglycan recycling [3], [4]. The ampC gene of *E.coli* is normally expressed at a low level but not induced as ampR is missing [5].

Plasmid mediated AmpC β -lactamases have arisen through the transfer of chromosomal genes of AmpC β -lactamases onto plasmids [6]. Plasmid-mediated AmpC β -lactamases have been found in *Klebsiella pneumoniae*, *Salmonella sp*, *Proteus mirabilis* and even *E. coli* and were first reported in 1988 [7].

Presently, all plasmid mediated AmpC β -lactamases have similar substrate profile to chromosomal AmpC β -lactamases. But the only difference is chromosomal AmpC β -lactamases are inducible where as plasmid mediated AmpC β -lactamases are uninducible [8].

AmpC β -lactamases confer resistance to cephalosporins in the oxyiminogroup (ceftazidime, cefotaxime, ceftriaxone, ceftizoxime, cefuroxime) and the 7- α -methoxy group (cefoxitin, cefotetan, cefmetazole, moxalactam) and monobactam (azotrenam) and are not affected by available β -lactamase inhibitors i.e. clavulanic acid, sulbactam etc.

AmpC β -lactamases producing organisms are on the rise and leads to therapeutic failure if 3rd Generation cephalosporins are given empirically or not tested in the laboratory for AmpC β -lactamases production. Specially, inducible AmpC β -

lactamases can be induced to several hundred folds higher in presence of clavulanic acid, where as clavulanic acid is commonly used as Extended spectrum β -lactamases (ESBL) inhibitor [9].

Aims & Objectives

The present study was undertaken:

1. To determine the prevalence of inducible AmpC β -lactamase producing *Pseudomonas aeruginosa* isolated in a rural hospital in Central India
2. To detect the antibiotic susceptibility pattern of *Pseudomonas aeruginosa* strains to newer antipseudomonal antibiotics.

Material And Methods

The study was conducted for a period of one year from 1st September 2006 to 31st August 2007. A total no of 244 *Pseudomonas aeruginosa* strains were isolated from different clinical specimens e.g. urine, pus, sputum, blood, endotracheal tube secretions and others in Microbiology laboratory of Jawaharlal Nehru Medical College, Wardha (M.S.) and were identified as per conventional methods [10]. Other specimens include throat swab, ear discharge, catheter tips, peritoneal fluid, vaginal swab, swab from buccal mucosa etc. Antibiotic susceptibility pattern of *Pseudomonas aeruginosa* isolates to newer antipseudomonal antibiotics e.g. Meropenem(10 μ g), Imipenem(10 μ g), Amikacin(30 μ g), ciprofloxacin(5 μ g), Piperacillin/Tazobactam(100/10 μ g), Azotrenam(30 μ g), Cefepime(30 μ g), Ceftazidime(30 μ g), Netilmicin(30 μ g), was detected by Kirby-Bauer disc diffusion method [11]. Antibiotics discs were procured from HiMedia Laboratories Pvt. Ltd, India. Screening of AmpC β -lactamase was done by disc antagonism test [12]. Confirmation of AmpC β -lactamase production was done by modified three dimensional test (MTDT) [13] and AmpC disc test [14].

Disc Antiagonism Test

In this test, lawn culture of test isolate (0.5 Mc Farland) was put over Muller-Hinton agar plate (MHA) and ceftazidime (30 μ g) and Cefoxitin (30 μ g) disc were placed 20mm apart from centre to centre. Plates were incubated for 18-20 hours at 37° C. AmpC β -lactamase inducibility was recognized by isolates showing blunting of ceftazidime zone of inhibition adjacent to cefoxitin disc and were considered screen positive [12][Table/Fig 1] .



(Table/Fig 1) Disc antiagonism test:Showing blunting of Cefotazidime zone of inhibition adjacent to Cefoxitin disc

Modified Three Dimensional Test (MTDT)

Confirmation of AmpC β -lactamase production was done by modified three dimensional test (MTDT) as described by Manchanda et al [13]. 10-15mg fresh overnight growth from Muller Hinton agar (MHA) was taken in a sterile 200 μ l ependroff tube.50 μ l peptone water was added and centrifuged at 800g for 15 minutes. Then by repeated freeze thawing for five to seven times, the crude enzyme extract was prepared. Lawn culture of *E. coli* ATCC 25922 was done in MHA plates and cefoxitin (30 μ g) disc was placed on the plate. A 3 cm linear slit was cut using a sterile scalpel blade 3 mm away from the cefoxitin disc. At the other end of the slit a well was cut. 30 μ l of crude enzyme extract was put in the well and plates were incubated.

Interpretation of MTDT:

Isolates showing clear distortion of zone of inhibition was taken as AmpC producers

[Table/Fig 2]. Isolates with no distortion of zone of inhibition was taken as AmpC nonproducers. Isolates with minimal distortion was taken as indeterminate strains.



(Table/Fig 2) MTDT test: showing clear distortion of zone of inhibition

AmpC Disc Test

AmpC β -lactamase production was further confirmed by AmpC disc test as described by Black et al [14].

Tris EDTA was used to permeabilize the bacterial cell and release of β -lactamase into external environment. AmpC discs were prepared in house. 20 μ l of 1:1 mixture of sterile saline and 100X Tris-EDTA was applied to sterile filter paper disc. The discs were allowed to dry and stored at 2-8° C. Before use, the AmpC discs were rehydrated with 20 μ l of sterile saline and 8-10 colonies of test strains was applied to a disc. A lawn culture of *E. coli* ATCC 25922 was done on Muller Hinton agar (MHA) plate. A Cefoxitin (30 μ g) disc placed on this inoculated MHA plate. The inoculated AmpC disc was placed almost touching the cefoxitin disc with inoculated disc surface in contact with agar surface. The plates were then incubated overnight at 37° C.

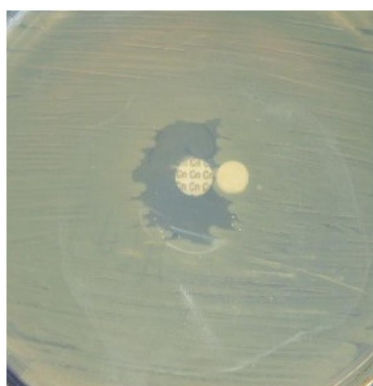
Interpretation Of AmpC Disc Test

Isolates showing either an indentation or a flatterring of the zone of inhibition indicating enzymatic inactivation of cefoxitin were taken as positive for AmpC production [Table/Fig 3],[Table/Fig 4].Isolates showing no distortion indicating no significant

inactivation of cefoxitin were taken as negative for AmpC production [14].



(Table/Fig 3) AmpC disc test: showing flattening of zone of inhibition



(Table/Fig 4) AmpC disc test: showing indentation of zone of inhibition

Results

Out of 244 *Pseudomonas aeruginosa* strains studied for inducible AmpC β -lactamase production 47 (19.3%) were positive by all the three methods used i.e. Disc antagonism test, [12] MTDT [13] and AmpC disc test [14][Table/Fig 5]. These 47 *Pseudomonas aeruginosa* strains were positive by disc antagonism test i.e. screen positive, [Table/Fig 1] which were further confirmed by MTDT [Table/Fig 2] and AmpC disc test [Table/Fig 3],[Table/Fig 4]. By MTDT 42 strains showed clear distortion of zone of inhibition and was taken as AmpC β -lactamase producer. 5 strains showed minimal distortion of zone of inhibition and was taken as indeterminate strains [Table/Fig 6] By AmpC disc test 39 strains showed indentation and 8 strains showed flattening of zone of inhibition. All 47 strains were taken as AmpC β -lactamase producer by AmpC disc test. The 5 indeterminate strains showing minimal

distortion also showed flattening in AmpC disc test indicating weak AmpC production [Table/Fig 6].

(Table/Fig 5) Prevalence of inducible AmpC β -lactamase producing *Pseudomonas aeruginosa* strains

| Total strains | Positive for AmpC β -lactamase | |
|---------------|--------------------------------------|------------|
| | Number | percentage |
| n= 244 | 47 | 19.3 |

(Table/Fig 6) Comparison of 3 different methods for AmpC β -lactamase production by *Pseudomonas aeruginosa*

| Screen positive (no) | Modified three dimensional test MTDT positive | | AmpC disc test positive | |
|----------------------|---|-------------------------|-------------------------|-----------------|
| | Distortion (no) | Minimal distortion (no) | Indentation (no) | Flattening (no) |
| 47 | 42 | 5 | 39 | 8 |

Out of these 244 *Pseudomonas aeruginosa* strains 218 (89.3%) were isolated from Indoor patients department (IPD) and 26 (10.7%) were from ICU patients. No *Pseudomonas aeruginosa* strain was isolated from Outdoor patients department (OPD) patients. Out of 47 AmpC β -lactamase producing *Pseudomonas aeruginosa* strains 2 (4.3%) were from ICU patients. Amongst these 2 ICU patients, from 1 (2.1%) patient, isolated *Pseudomonas aeruginosa* strain was resistant to all anti-pseudomonal antibiotics studied. Another *Pseudomonas aeruginosa* strain isolated from ICU patient was also resistant to ciprofloxacin, piperacillin/tazobactam, aztreonam, cefepime, ceftazidime and netilmicin.

The *Pseudomonas aeruginosa* strains isolated from different specimens were urine 82 (33.6%), pus 63 (25.9%), sputum 37 (15.1%), blood 12 (5%), endotracheal tube secretion 7 (2.9%), and others 43 (17.6%). Out of 47 AmpC β -lactamase producing *Pseudomonas aeruginosa* strains 21 (44.7%) strains were isolated from urine followed by 12 (25.5%) from pus and 8 (17%) from sputum respectively [Table/Fig 7]. A total number of 225 (92.2%) *Pseudomonas aeruginosa* strains were sensitive to meropenem and imipenem, followed by 201

(82.4%) strains sensitive to amikacin and 184 (75.4%) strains sensitive to ciprofloxacin [Table/Fig 8]. The highest sensitivity pattern observed was Imipenem, Amikacin, and Ciprofloxacin. 19 (7.8%) *Pseudomonas aeruginosa* strains were resistant to all 9 newer antipseudomonal antibiotics studied, of which 3 (15.8%) strains were AmpC β -lactamase producer.

(Table/Fig 7) Prevalence of AmpC β -lactamase producing strains isolated from different clinical specimens (n=244)

| Specimen | Number | AmpC β -Lactamase+ve | |
|-----------------------------|--------|----------------------------|------------|
| | | Number | percentage |
| Urine | 82 | 21 | 25.6 |
| Pus | 63 | 12 | 19 |
| Sputum | 37 | 8 | 21.6 |
| Blood | 12 | 2 | 16.7 |
| Endotracheal Tube secretion | 7 | 1 | 14.3 |
| Others | 43 | 3 | 7 |
| Total | 244 | 47 | 19.3 |

Others include throat swab, ear discharge, catheter tips, peritoneal fluid, vaginal swab, swab from buccal mucosa etc.

(Table/Fig 8) In vitro susceptible pattern of *Pseudomonas aeruginosa* isolates (n= 244)

| Antibiotics | Susceptible <i>Pseudomonas aeruginosa</i> | |
|-------------------------|---|------------|
| | Number | percentage |
| Meropenem | 225 | 92.2 |
| Imipenem | 225 | 92.2 |
| Amikacin | 201 | 82.4 |
| Ciprofloxacin | 184 | 75.4 |
| Piperacillin/Tazobactam | 175 | 71.7 |
| Azotrenam | 165 | 67.6 |
| Cefepime | 161 | 66 |
| Ceftazidime | 156 | 63.9 |
| Netilmicin | 120 | 49.2 |

Discussion

AmpC β -lactamase producing bacteria can cause major therapeutic failure if they remain undetected because in routine Kirby-Bauer Disc diffusion test for antibiotic sensitivity, they may show false sensitivity zone. But the facts is AmpC β -lactamase producing organisms particularly *Pseudomonas aeruginosa* is on the rise and poses a major therapeutic challenge due to treatment failure [15], and have been responsible for several nosocomial outbreak. The major threat is that *E.coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Salmonella* species can produce plasmid mediated AmpC β -lactamase making most bacterial strains resistant to ceftaxime and other cephalosporins, But most Clinical laboratories and Physicians remain unaware

of the clinical importance of AmpC β -lactamase producing organism [16]. The detection of Extended spectrum β -lactamases (ESBL) in AmpC producing species of Gram negative bacteria is another major problematic area. High level expression of AmpC may prevent recognition of an ESBL specially if inhibitor based approach i.e. clavulanic acid is taken to detect ESBL. The reason is— clavulanic acid may acts as inducer of high level AmpC production in those organisms e.g. *Pseudomonas aeruginosa*, *Serratia*, *Enterobacter sp.* etc, which can produce chromosomally encoded inducible AmpC β -lactamase [9].

But there is a paucity of data regarding inducible AmpC β -lactamase producing *Pseudomonas aeruginosa*, not only in India but Worldwide also. No Clinical & Laboratory Standards Institute (CLSI) recommendation exists for phenotypic screening or confirmatory tests for inducible AmpC β -lactamase producing bacteria [17]. Current detection methods for detecting AmpC β -lactamase producing organisms are technically demanding. Though multiplex PCR has been done, it is not yet available for routine use [16]. In the present study we had 19.3% of our *Pseudomonas aeruginosa* strains which produce AmpC β -lactamase. Our study correlated well with reports from Aligarh by Shahid et al. in 2004 as 20% [17] and from Kolkata by Arora et al. in 2005 as 17.3% [15] and from Varanasi as 22% [18]. Bhattacharjee et al. from IMS, BHU has reported that 94% of their *Pseudomonas aeruginosa* strains were sensitive to Piperacillin/ Tazobactam [18] but in our study only 71.7% strains were sensitive to piperacillin / Tazobactam. In the present study maximum no of strains (92.2%) were sensitive to Meropenem and Imipenem compared to only 86% sensitivity to imipenem in the study conducted in BHU [18].

Amongst the 19 *Pseudomonas aeruginosa* strains resistant to all newer antipseudomonal antibiotic studied, 3

(15.8%) strains were AmpC β -lactamase producer, and 16 (84.2%) were resistant to all antipseudomonal antibiotics but AmpC β -lactamase negative.

Modified three dimensional test (MTDT) is not feasible to do routinely as it is cumbersome for confirmation of AmpC β -lactamase producer. Compared to MTDT, all (100%) *Pseudomonas aeruginosa* strains producing AmpC β -lactamase could be detected by AmpC disc test in our study. It should be clarified here that though AmpC disc test was originally introduced to detect plasmid mediated AmpC β -lactamase, Black et al. reported that AmpC disc test detect high level production of chromosomally mediated inducible AmpC β -lactamase in *Pseudomonas aeruginosa*, *Acinetobacter species*, *Enterobacter cloacae* etc [14].

Conclusion

Though specific CLSI guideline is till not available for detection of AmpC β -lactamase, clinical Microbiologists should start screening of AmpC β -lactamase producing bacterial strains to prevent misuse of antibiotics and therapeutic failure. Amongst the different methods available, AmpC disc test is quite easy which can be routinely done to detect AmpC β -lactamase production in any Microbiology Laboratory set up.

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