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Research Article

PHENOTYPIC DETECTION OF AmpC β-LACTAMASE PRODUCTION IN GRAM NEGATIVE CLINICAL ISOLATES

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Abstract- Background: Detection of AmpC β-lactamase producing isolates is needed to provide the accurate and effective treatment to patient for their better outcome. In laboratories test can be performed by simple phenotypic screening and confirmation method. **Objectives:** To determine the prevalence of MDR isolates and AmpC β-lactamase producers from clinical samples. **Materials and Methods:** A total no. of 600 gram negative organisms included in the study from which 318 were MDR. All MDR isolates screened and confirmed for AmpC β-lactamase production by cefoxitin and Cefoxitin, cefoxitin-cloxacillin double disc synergy test respectively. For inducible AmpC β-lactamase production cefotaxime and cefoxitin discs were used. Blunting of zone of inhibition of cefotaxime placed adjacent to cefoxitin considered positive for inducible AmpC producer. **Results:** From total 600-gram negative organisms, 318 were MDR gram negative isolates. Out of 318 gram negative isolates, 281 showed positive AmpC β-lactamase screening test by cefoxitin resistance. Prevalence of AmpC β-lactamase producers was 42.70% from screening positive isolates. **Interpretation and conclusion:** Accurate detection of AmpC β-lactamase producers in laboratories and proper treatment of patient will help to control the spread of these pathogens and control of antibiotic resistance.

Keywords- Prevalence, AmpC β- lactamase, MDR isolates.

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Introduction

Development of multidrug resistant gram negative organisms has a global impact on increased incidence of infections. This resistance is mainly due to the enzymes produced by these microorganisms which hydrolyze the β-lactam antibiotics [1,2]. AmpC β-lactamases are cephalosporinases conferring resistance to cephamycins (cefoxitin and cefotetan), another extended spectrum cephalosporins, aztreonam and resist inhibition by clavulanate, sulbactam and tazobactam [3-6]. Microorganisms producing these enzymes may appear sensitive to extended spectrum cephalosporins when tested in laboratory initially and become resistant upon therapy. Therefore, false susceptibility report of cephalosporins and prescription of inappropriate antimicrobial regimen to patient can lead to therapeutic failure [1,7].

AmpC β-lactamases belong to Group 1 according to Bush *et al.* functional classification scheme, while in Class C in the Ambler's structural classification. The molecular classification divides β-lactamase enzyme types based on sequence of amino acid in the proteins and numerical groups based on hydrolysis or inactivating properties of enzymes for important class of β-lactams [8].

These β-lactamases are plasmid mediated and chromosomal. Plasmid mediated AmpC genes due to their mobility easily spread in genus, species and to different organisms [3,9]. In many gram-negative organisms, chromosomal AmpC genes are expressed at higher level as a consequence of mutation. Sometimes these enzymes expressed are at low level and can be induced in response to β-lactam exposure. Imipenem and cefoxitin are strong inducers while weak inducers are cefotaxime, ceftriaxone, ceftazidime, piperacillin and aztreonam. Beta-lactamase inhibitors especially clavulanic acid also act as inducing agent. Clavulanic acid has less inhibitory effect on AmpC β-lactamase enzyme but gradually increases AmpC mediated resistance in an inducible microorganism [3,8].

Due to multidrug resistance, limitations in therapeutic options, emergence of more strains, it is necessary to know the accurate prevalence of AmpCβ-lactamase producing isolates. Therefore, present study was taken to determine the prevalence of AmpC β-lactamase producing strains in multidrug resistant (MDR) organisms which were also resistant to β-lactam inhibitor clavulanic acid and/or tazobactum in a tertiary care hospital.

Materials and Methods

This was a prospective observational study carried out in the Department of Microbiology, Dr D.Y. Patil Medical College, Hospital and Research Centre, Dr. D.Y Patil Vidyapeeth, Pimpri, 411018, Pune. The approval to conduct this study was obtained from the Institute Ethics Committee.

A total no. of 600-gram negative isolates from various samples such as blood, urine, sputum, pus, and other body fluids received from various hospitalized patients and outpatient department included in the study.

For isolation all samples except urine were inoculated on Blood and MacConkey agar plates. Urine samples inoculated on CLED (Cysteine Lactose Electrolyte Deficient) agar plates. The growth on agar plates identified to genus and species level by standard biochemical reactions.

Antimicrobial susceptibility testing was performed by Kirby Bauer disc diffusion method for norfloxacin (10µg), ceftazidime (30µg), cephotaxime (30 µg), imipenem (10 µg), amikacin (30µg), gentamicin (10µg), ampicillin (10µg), piperacillin (100µg), nitrofurantoin (300 µg), nalidixic acid (30µg), cotrimoxazole (25µg), chloramphenicol (30µg), ciprofloxacin (5µg). Interpretation of zone size was done according to CLSI guidelines [10].

Out of 600 isolates which were MDR (resistant to at least one agent in three or more categories of drugs) [11,12] and resistant to β-lactamase inhibitors (clavulanic acid and or tazobactam) were included. Screening and confirmatory

test for AmpC detection carried out by disc diffusion method.

Screening criteria for suspecting an isolate as AmpC producer
Resistant to cefoxitin (30µg)-Zone diameter less than 18mm [6,7,9,13,14].

Disc antagonism test
This test was performed for detection of inducible AmpC β- lactamase producer. Disc of cefotaxime (30µg) and cefoxitin (30µg) were placed 20mm apart from center to center on Muller Hinton agar plates with lawn culture of test isolate. Isolate showing blunting of zone of inhibition of cefotaxime disc placed adjacent to the inducer cefoxitin disc considered as positive for inducible AmpC β-lactamase [Fig-1] [6,13,14,15].

Confirmatory test for AmpC β-lactamases (cefoxitin- cefoxitin cloxacillin double disc synergy test-[CC-DDS]) [1, 14]
The test is based on inhibitory effect of cloxacillin on AmpC enzyme. Disc containing cefoxitin (30 µg) and cefoxitin-cloxacillin (30/200 µg) was used. A difference in the inhibition zone of cefoxitin-cloxacillin minus the cefoxitin alone of ≥ 4mm considered positive for AmpC production [Fig-2].

Results
Out of 600 (100%) gram negative isolates tested, 318 (53%) were multidrug resistant including resistance to β-lactamase inhibitor. Of these 318 gram negative organisms 37(11.63%) were sensitive to cefoxitin and 281(88.36%) strains resistant to cefoxitin (AmpC screening positive strain). These 281 isolates were further processed for phenotypic confirmation test for AmpC-β-lactamase production. 120 (42.70%) strains were phenotypically confirmed as AmpC-β-lactamase producers. *K. pneumoniae* and *E. coli* strains were predominant AmpC-β-Lactamase producers, 40(33.33%) and 38 (31.66%) respectively. Percentages of other AmpC producers were *Citrobacter sps*. 14 (11.66%), *Acinetobacter sps.* 13 (10.83%), *P.aeruginosa* 11(9.16), other nonfermenters 3(2.5%) and *Proteus mirabilis* 1(0.83%) [Table-1].

Table-1 *Various organisms producing AmpC β-lactamase enzyme*

Organisms	AmpC screening positive isolates n=281 (%)	Phenotypic confirmation of AmpC producers n=120 (%)
K. pneumonia	89 (31.67)	40 (33.33)
E. coli	84 (29.89)	38 (31.66)
P. aeruginosa	48 (17.08)	11 (9.16)
Acinetobacter sps.	28 (9.96)	13 (10.83)
Citrobacter sps.	24 (8.54)	14 (11.66)
Other Nonfermenters	5 (1.77)	3 (2.5)
P.mirabilis	2 (0.71)	1 (0.83)
Enterobacter sps.	1 (0.39)	0 (0)

Maximum percentage of AmpC β-lactamase producer isolates were from pus sample 37/120 (30.83%) followed by urine sample 27/120(22.5%). AmpCβ-lactamase producing strains from respiratory specimen and catheter tips were 14/120 (11.66%) separately while from blood and body fluids were 17/120(14.16%) and 11/120 (9.16%) respectively [Table-2].

Table-2 *Distribution of AmpC screening positive and AmpC producer isolates from clinical specimens*

Clinical sample	AmpC screening Positive isolates n=281 (%)	Phenotypic confirmation of AmpC producers n=120 (%)
Pus	89 (31.67)	37 (30.83)
Urine	59 (20.99)	27 (22.5)
Respiratory specimen	39 (13.87)	14 (11.66)
Catheter tips	34 (12.09)	14 (11.66)
Blood	32 (11.38)	17 (14.16)
Body fluids	28 (9.96)	11 (9.16)

Of the total AmpC β-lactamase producers; 47(39.16%) strains were isolated from patients admitted in surgery ward while 27 (22.5%) strains were isolated from various ICUs (MICU, SICU, PICU, NICU).
AmpC-β-lactamase producer strains isolated from samples received from other wards were 15 (12.5%) from medicine ward,9 (7.5%) from pulmonary ward, 8 (6.66%) from paediatrics, 7(5.83%) from gynaecology ward, 3(2.5%) from orthopedics ward,3(2.5%) from OPD and 1(0.8%) from psychiatric ward [Table-3].

Table-3 *Ward wise distribution of AmpC β-lactamase producer isolates*

Ward	AmpCscreening Positive isolates n=281 (%)	Phenotypic confirmation of AmpC producers n=120(%)
Surgery	100 (35.58)	47 (39.16)
Intensive Care Units (ICUs)	92 (32.74)	27 (22.5)
Medicine	29 (10.32)	15 (12.5)
Pulmonary Medicine	15 (5.33)	9 (7.5)
Gynaecology	13 (4.62)	7 (5.83)
OPD	12 (4.27)	3 (2.5)
Paediatrics	10 (3.55)	8 (6.66)
Orthopedics	9 (3.20)	3 (2.5)
Psychiatric	1 (0.35)	1 (0.83)

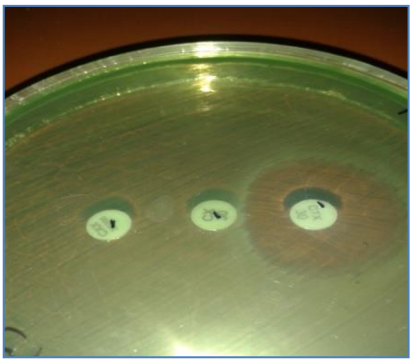

Fig-1 Detection of inducible AmpC β-lactamases (Disc antagonism test)

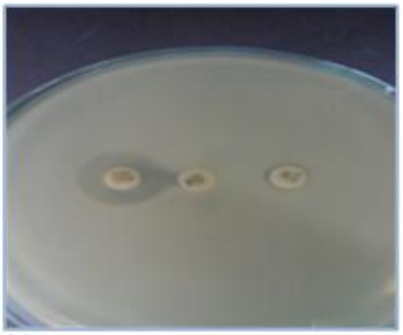

Fig-2 Detection of AmpC β-lactamase (CC-DDS)

Out of 120 AmpCβ-lactammase producers, 14 (11.66%) were inducible AmpCβ-lactamase producers [Fig-3]. Most of the inducible AmpC β-lactamase producers were maximum *Pseudomonas aeruginosa*isolates9/14(64.28%) [Fig-3]. Out of 14 inducible AmpC β-lactamase producer isolates, 9 (64.28%) were from surgery ward of which 6/9(55.55%) were *P. aeruginosa.*

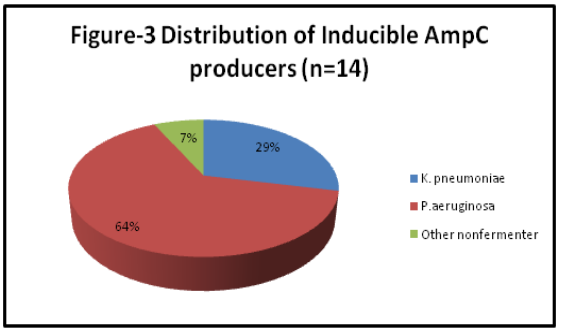

Fig-3 Distribution of Inducible AmpC producers

Discussion

E. coli, Klebsiella sps. and P. aeruginosa are the most commonly isolated species among the gram-negative organisms in the clinical laboratory. However, very few studies have been reported in the prevalence of AmpC-β lactamases among these species. In this study, 53% isolates were multidrug resistant strains that are resistant to β- lactamase inhibitors *i.e*., clavulanic acid and tazobactam also. In a study of Afunva RA et al they showed the percentage of 90% from community and 30% from hospital isolated strains were resistant to amoxicillin clavulanic acid [16]. In this study, prevalence of AmpCβ-lactamase producer isolates from total gram-negative isolates was 20%, from MDR gram negative isolates was 37.73% and 42.70% from AmpCβ-lactamase screening positive isolates by using cefoxitin-cefoxitin cloxacillin double disc synergy test for confirmation. Hemalatha, *et al*., 2017, in his study found 47.3% and Khan, *et al*., 2015, 22% isolates AmpCβ-lactamase producers [17,18]. In Mohamudha, *et al*., 2010, study plasmid mediated AmpCβ-lactamase producers by AmpC disc test was detected in 80.9% of screen positive isolates and 93.6% producers by using three-dimensional enzyme extraction method [5]. The occurrence of increasing reports of AmpCβ-lactamase producing organisms leading to therapeutic failure due to resistance to a wide variety of β-lactam drugs and plasmid mediated transferable resistance mechanism poses a challenge for laboratories to detect them.

Maximum percentage of AmpCβ-lactamase producer isolates in this study were *K.pneumoniae* (33.33%), followed by *E.coli* (31.66%) and *Citrobacter sps*. (11.66%). In recent years, a significant increase in ESBL Producing *K.pneumoniae* in hospitalized patients are problematic throughout world [19]. In a study of Rajni, *et al*., 2008, maximum incidence of AmpCβ-lactamase producers were seen among *E.coli* (70%) followed by *K. pneumoniae* (56.7%) [20]. However, Manchanda and Singh from Delhi found higher rates of AmpCβ-lactamase production among *K. pneumoniae* (33.3%) and *P. mirabilis* (33.3%) isolates, but a lower rate among *E. coli* isolates (14.3%) [21]. In a study of Laghawe, *et al*., 2012, maximum AmpC producers were *Citrobacter freundii* (62.5%), followed by *Enterobacter cloacae* (52.63%) and in Nagdeo, *et al*., 2012, study maximum AmpC β-lactamase producers were *Klebsiella sps.* (84.16%) followed by non-fermenters (60%) [13,15]. The Occurrence of *Acinetobacter* spp. after pseudomonas among non-fermenter is high in hospital [22]. Different prevalence of AmpCβ-lactamase producers in different studies may be due to differences in geographical distribution giving rise to varied resistance pattern.

We isolated maximum percentage of AmpCβ-lactamase producer microorganisms from pus samples (30.83%) followed by urine samples (22.5%). Majority of AmpCβ-lactamase producer isolates from urine (52.6%) followed by respiratory tract (18.4%) found by Polsfuss, *et al*., 2011, [1]. In a study of Madumati, *et al.,* 2015, highest percentage of Amp C was reported from pus (61%), followed by tracheal aspirates samples (48%) [23].

Surgery ward (39.16%) and ICUs (22.5%) isolates have maximum AmpC producer isolates. In a study of Neha, *et al*.,2014, the frequency of the maximum AmpC producer organisms were from orthopaedics wards, followed by surgery wards, medicine ward, ICU [24].

Majority of inducible AmpC β- lactamase producers from this study were *Pseudomonas aeruginosa* (64.28%). In Laghawe, *et al*., 2012, study also maximum inducible strains belong to *Psedomonas aeruginosa* (77.7%) [13]. Treatments of inducible strains are more difficult due to their false susceptibility to cephalosporins and uncontrolled spread. Therefore, in hospital setting, these should be identified quickly. In this study, maximum inducible isolates were from surgery ward. Ward distribution in hospital setting helps to know the drug resistance pattern in different areas and if needed to isolate the patient and prevent the transfer of drug resistance. It also helps to trace the source of drug resistant strain.

Phenotypic tests cannot distinguish among the various families of plasmid - mediated AmpC enzymes and may also overlook chromosomally determined AmpC β-lactamases with an extended spectrum. For, these purposes, detection of AmpC β-lactamase by multiplex PCR are gold standard.

Conclusion

Regular monitoring of AmpC producers is necessary to prevent their spread in hospital and community, prevent treatment failure and mortality in patients. AmpC disc test is easier and rapid test can be used for routine screening and confirmation of this enzyme in the microbiology laboratories.

Application of research: Accurate detection of AmpC β-lactamase producers in laboratories and proper treatment of patient will help to control the spread of these pathogens and control of antibiotic resistance.

Research Category: Multidrug resistant gram-negative organisms

Abbreviations:
MDR: Multidrug Resistant
ESBL: Extended-spectrum β-lactamases

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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