

AMPC BETA LACTAMASES AMONG GRAM NEGATIVE CLINICAL ISOLATES FROM A TERTIARY HOSPITAL, SOUTH INDIA

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ABSTRACT

AmpC β -lactamases are cephalosporinases that hydrolyze cephamycins as well as other extended-spectrum cephalosporins and are poorly inhibited by clavulanic acid. Although reported with increasing frequency, the true rate of occurrence of AmpC β -lactamases in different organisms, including members of *Enterobacteriaceae*, remains unknown. The present study was designed to determine the occurrence of AmpC enzyme-harboring Gram-negative clinical isolates in a tertiary care hospital in Pondicherry state, South India. A total of 235 Gram negative clinical isolates were tested for resistance to ceftiofuran, third generation cephalosporin (3GC) antibiotics, ampicillin, amikacin, co-trimoxazole, gentamicin, meropenem and tetracycline by disc diffusion method. Isolates found resistant to 3GC and ceftiofuran were tested for the production of AmpC β -lactamases by three dimensional extraction method and AmpC disc method. Isolates found to sensitive to 3GC were subjected to disc antagonism test for inducible AmpC production. One hundred and thirty four (57%) strains were resistant to 3GC, among which 63(47%) were positive for plasmid-mediated AmpC beta lactamases production. Among the 101 strains sensitive to 3GC, 23 (22.7%) revealed the presence of inducible AmpC beta lactamases by disc approximation test. A total of 80.9% (51/63) of screen positive isolates were detected by Amp C disc test and 93.6% (59/63) by three dimensional extraction method. Out of the 86 AmpC producers, 67 (77.9%) were ceftiofuran resistant. Inducible AmpC was not found in *Esch.coli* and *Klebsiella spp.* The AmpC producers also concurrently showed multidrug resistance pattern. AmpC producers were found to be prevalent in our hospital and though three dimensional extraction test detects AmpC better, the disk test is easier to perform routinely and is user- friendly.

Key words: Gram negative organisms, drug resistance, three-dimensional technique, AmpC β -lactamases

INTRODUCTION

The predominant mechanism for resistance to β -lactam antibiotics in Gram-negative bacteria is by the synthesis of β -lactamases (23). Among the β -lactamases the production of ESBLs and AmpC β -lactamases are the most common (10).

AmpC β -lactamases are class C or group I cephalosporinases that confer resistance to a wide variety of β -lactam antibiotics including alpha methoxy β -lactams such as ceftiofuran, narrow and broad spectrum cephalosporins, aztreonam, and are poorly inhibited by β -lactamase inhibitors such as clavulanic acid (8). Chromosome-mediated AmpC β -lactamases have been

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described in a wide variety of Gram-negative bacilli, such as *Pseudomonas aeruginosa* and *Enterobacter spp.*, *Acinetobacter spp.*, *Aeromonas spp.*, *C. freundii*, *E. coli*, *S. marcescens*, etc. Overproduction of their chromosomal AmpC β -lactamase by mutation is probably responsible for the resistance in these organisms (13, 18). In most genera of the family *Enterobacteriaceae*, AmpC is inducible (8). Unlike chromosome-mediated AmpC, plasmid-encoded AmpC enzymes are almost always expressed constitutively (18). Many plasmid-mediated AmpC enzymes, such as CMY-type β -lactamases, have been found in bacterial species that naturally lack a chromosomal AmpC β -lactamase, such as *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Salmonella spp.* It is believed that such β -lactamases arose through the transfer of chromosomal AmpC genes onto plasmids. Plasmid-mediated inducible β -lactamases are extremely rare (11).

Several epidemiological studies have shown that AmpC enzyme producing bacteria are recovered from hospitalized patients after several days of admission to the hospital. Affected patients have often had prolonged stay. A majority of patients had been treated with β -lactam antibiotics including cefoxitin (16). Although it has been over a decade since plasmid-mediated AmpC β -lactamases were discovered, most clinical laboratories and physicians remain unaware of their clinical importance (2). As a result, organisms producing these types of β -lactamases often go undetected and therefore have been responsible for several nosocomial outbreaks (18). Without accurate laboratory detection and reporting of such resistant phenotypes and strains producing plasmid-mediated AmpC, treatment of Gram negative infection may remain suboptimal.

There is a paucity of information on the documentation of AmpC β -lactamases among Gram negative isolates this region. This is due in part to the lack of standard guidelines for detecting AmpC-producing isolates. Since, the detection of organisms producing these β -lactamases is thus important; a prospective study was undertaken to determine the occurrence of AmpC β -lactamases in Gram negative clinical isolates at this centre.

MATERIALS AND METHODS

Patients and bacterial strains

This study was carried out in a 750-bedded, urban, tertiary care hospital between May to July 2008. A total of 235 consecutive clinical isolates of *E. coli* (n=65), *K. pneumoniae* (n=38), *Enterobacter* (n=26), *Acinetobacter* (n=12), *Pseudomonas* (n=48), *Citrobacter* (n=10), *Proteus* (n=31), *Morganella morganii* (n=4) and *Providencia* (n=1) were obtained from various clinical specimens such as blood, urine, sputum, ascitic fluid and pus of hospitalized patients (one isolate per patient). All the isolates were identified biochemically by the standard methods (14) and were stored at 4°C in 0.2% semisolid agar until used.

Antimicrobial susceptibility testing

The antibiogram of the isolates were determined by the standard Kirby Bauer's disc diffusion method (3). The following antibiotics discs (Hi-Media, India) were used such as, ampicillin (10 μ g), amikacin (30 μ g), gentamicin (10 μ g), co-trimoxazole (25 μ g), ciprofloxacin (5 μ g), cefotaxime (30 μ g), ceftazidime (30 μ g), ceftriaxone (30 μ g), cefoxitin (30 μ g) and meropenem (10 μ g). The zone diameters were interpreted as per Clinical Laboratory Standards Institute (CLSI) recommendations (9). *Escherichia coli* ATCC 25922 strain was used for quality control.

Detection of Inducible AmpC Production

Disc antagonism test (DAT): Isolates that fell in the sensitivity range to cefotaxime, ceftazidime or ceftriaxone (3GC) and cefoxitin as per the CLSI were subjected to disc antagonism test for inducible AmpC detection by the method of Sanders *et al.* (20).

Detection of Plasmid-mediated AmpC production

AmpC disk test: Isolates that yielded a cefoxitin zone diameter less than 18 mm and resistant to 3GC (screen positive) were tested for AmpC enzyme production by AmpC disk test (7). Briefly, 0.5 McFarland suspension of ATCC *E.*

coli 25922 was inoculated on the surface of Mueller-Hinton agar plate. A 30 µg cefoxitin disc was placed on the inoculated surface of the agar. A sterile plain disc inoculated with several colonies of the test organism was placed beside the cephoxitin disc almost touching it, with the inoculated disk face in contact with the agar surface. After overnight incubation at 37°C, the plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cefoxitin (positive result), or the absence of a distortion, indicating no significant inactivation of cefoxitin (negative result).

Modified three-dimensional test: Plasmid-mediated AmpC beta lactamases production was further confirmed by the modified three-dimensional test (15). Briefly, fresh overnight growth of test organism from Mueller-Hinton agar plate was taken in a micro centrifuge tube. Peptone water was added and centrifuged at 3000 rpm for 15 min. Crude enzyme extract was prepared by repeated freeze thawing in -80°C for seven times. A lawn culture of *E.coli* ATCC 25922 was prepared on MHA plates and cefoxitin (30 µg) discs were placed on the plate. Linear slits were cut using a sterile surgical blade 3 mm away from the cefoxitin disc; 30 to 40 µl of the enzyme extract was added to a well made at the outer edge of the slit, without overflowing. The plates were kept upright for 5 to 10 minutes until the liquid dried and were incubated at 37°C for overnight. Clear distortion of zone of inhibition of cephoxitin is taken as Amp C beta- lactamase producers.

RESULTS

Antimicrobial susceptibility testing

Out of the 235 total isolates tested, 134 (57%) were resistant to 3GC (cefotaxime, ceftazidime, ceftriaxone), while 101 (42.9%) strains were susceptible. Majority of the *Klebsiella* and *E.coli* and *Enterobacter* isolates showed multidrug resistance. They were resistant to at least one non-β-lactam antibiotic (amikacin, gentamicin, co-trimoxazole, and

tetracycline). All of the *Providencia* and *Morganella* tested were susceptible to all the antibiotics used. None of the isolates were resistant to meropenem.

Inducible AmpC Production

Among the 101 sensitive strains, 23 (22.7%) revealed the presence if inducible AmpC beta- lactamases by disc antagonism test (Fig.1). A maximum number of 42.8% of *Pseudomonas* spp. were inducible AmpC producers followed by *Enterobacter* spp. and *Proteus* 41.6%, 31.5% respectively. None of the *E.coli*, *Klebsiella* or *Acinetobacter* showed inducible AmpC positive. Table 1 shows the distribution of AmpC producers among the different clinical isolates.

Plasmid-mediated AmpC production

Of the 134 third generation cephalosporin resistant strains, 63(47%) were positive for plasmid-mediated AmpC beta lactamases. 75% of *Proteus* were stable AmpC producers followed by *Acinetobacter* and *Citrobacter* 66.6% each respectively.

Plasmid-mediated AmpC β-lactamase production was detected in 80.9% (51/63) of screen positive isolates by Amp C Disc test (Fig.2) and 93.6% (59/63) by three dimensional enzyme extraction method. Of the 86 total AmpC producers, 67 (77.9%) were cefoxitin resistant and 19 (22%) were cefoxitin sensitive, shown in table 2.

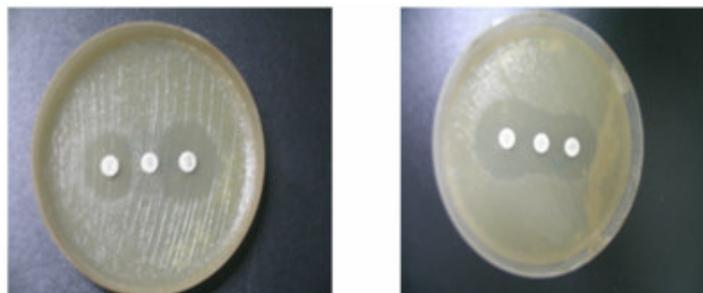


Figure 1. Disk antagonism test. Blunting of the cephalosporin disk adjacent to the cefoxitin disk, positive (A), absence of blunting indicates negative (B).

Table 1. Prevalence and distribution of AmpC producing Gram negative clinical isolates.

Organism	Total No. of organisms isolated	Isolates susceptible to 3GC	Inducible AmpC		Isolates resistant to 3GC	Plasmid-mediated AmpC	
			Positive	Negative		Positive	Negative
<i>E.coli</i>	65	22 (33.8)	-	22 (100)	43(66.1)	11(25.5)	32(74.4)
<i>Klebsiella</i>	38	15 (39.4)	-	15(100)	23(60.5)	9(39.1)	14(60.8)
<i>Acinetobacter</i>	12	3 (25)	-	3 (100)	9(75)	6(66.6)	3(33.3)
<i>Pseudomonas</i>	48	21(43.7)	9(42.8)	12(57.1)	27(56.25)	15(55.5)	12(44.4)
<i>Enterobacter</i>	26	12 (46.1)	5(41.6)	7(58.3)	14(53.8)	9(64.2)	5(35.7)
<i>Citrobacter</i>	10	4 (40)	1(25)	3(75)	6(60)	4 (66.6)	2(33.3)
<i>Proteus</i>	31	19 (61.2)	6(31.5)	13(68.4)	12(38.7)	9(75)	3 (25)
<i>Morganella</i>	4	4 (100)	1(25)	3(75)	-	-	-
<i>Providencia</i>	1	1 (100)	1 (100)	-	-	-	-

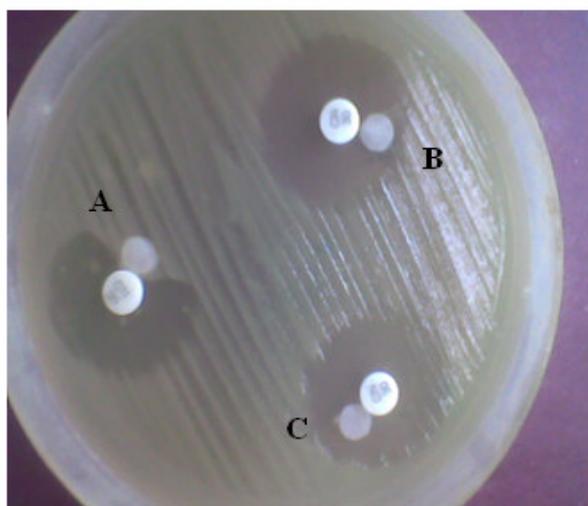


Figure 2. AmpC disk test: Presence of blunting towards cefoxitin disk indicates test positive (A) absence of blunting indicates test negative (B and C).

Table 2. Frequency of cefoxitin resistance among AmpC positive

Total AmpC positive isolates (n)	cefoxitin resistant (n)	cefoxitin susceptible (n)
<i>E.coli</i> (11)	9	2
<i>Klebsiella</i> (9)	8	1
<i>Acinetobacter</i> (6)	6	-
<i>Pseudomonas</i> (24)	18	6
<i>Enterobacter</i> (14)	10	4
<i>Citrobacter</i> (5)	5	-
<i>Proteus</i> (15)	9	6
<i>Morganella</i> (1)	1	-
<i>Providencia</i> (1)	1	-

DISCUSSION

Genes for AmpC β -lactamases are commonly found on the chromosomes of the several members of the family Enterobacteriaceae, including *Enterobacter*, *Shigella*, *Providencia*, *Citrobacter freundii*, *Morganella morganii*, *Serratia marcescens* and *Escherichia coli*. Plasmid mediated AmpC β -lactamases has arisen through the transfer of chromosomal genes for the inducible AmpC β -lactamases on to plasmids. This transfer has resulted in plasmid mediated AmpC β -lactamases in isolates of *E.coli*, *Klebsiella pneumoniae*, *Salmonella* species, *Citrobacter freundii*, *Enterobacter aerogenes* and *Proteus mirabilis* (17). Plasmid mediated AmpC β -lactamases represent a new threat since they confer resistance to cephamycins and are not affected by β -lactamase inhibitors, and can, in strains with loss of outer membrane porins, provide resistance to carbapenems. This resistance mechanism has been found around the world, can cause nosocomial outbreaks, appears to be increasing in prevalence, and merits further study to define the best options for detection and treatment (18). Although plasmid-mediated AmpC β -lactamases were first reported in the late 1980s, many infectious disease personnel remain unaware of their clinical importance. The number of infections caused by AmpC producing organisms is increasing (4, 6, 11). Distinguishing between AmpC- and ESBL-producing organisms has epidemiological significance and has therapeutic importance as well. Plasmid mediated AmpC β -lactamases from *K.pneumoniae* isolates was first reported in 1989 from Seoul, South Korea (5). From US, 6.9 % of *K.oxytoca* and 4% of *Esch. coli* were AmpC producers (1). The prevalence of AmpC β -lactamases in *K. pneumoniae* was 17.1 %, *E. Coli* 2% in China (25) DHA-1 type of inducible AmpC β -lactamases were first reported in *Salmonella enteritidis* from Saudi Arabia in 1998 (2). Plasmid mediated inducible AmpC β -lactamases still rare. So far no reports of inducible AmpC among *Esch.coli* and *Klebsiella spp* from India also. From North India, 20 % of *P. aeruginosa* (Delhi) and 20.7 % of Gram-negative organisms (Aligarh) and 47.8% *E.coli*, 17.3%

P. aeruginosa, 13% *K. pneumoniae* (Kolkota) were reported as AmpC β -lactamase producers (15, 21, 22). From South Indian states, 24.1 % of *Klebsiella* spp. and 37.5 % of *E. coli* were AmpC producers from Chennai; In Karnataka, 3.3 % of *E. coli*, 2.2 % of *K. pneumoniae*, 5 % of *C. freundii*, and 5.5 % *E. aerogenes* were found to harbour AmpC enzymes (24, 19). In the present study, 22% of AmpC producers were found to be susceptible to cefoxitin. AmpC production in these isolates could be due a mechanism similar to that of ESBL producing organism that appear susceptible to ceftazidime by the disc diffusion method. They may also have MIC of cefoxitin <2 μ g/ml, which was not tested. This finding was similar to a previous study, which reported 39% of cefoxitin susceptibility in AmpC producers (15).

Cefoxitin resistance in AmpC non-producers could be due to some other resistance mechanism such as, lack of permeation of porins (12). Inducible expression of chromosomal AmpC β -lactamases, although rare in *E. coli* and *K. pneumoniae*, is associated with a significant risk of therapeutic failure with all β lactam drugs except carbapenems (18). Hence, an attempt was made not to fail to identify inducible strains by DAT. None of the isolates were positive for inducible AmpC β -lactamase thus revealing the presence of only plasmid mediated resistance in *Klebsiella* and *Esch.coli* in them. No resistance to carbapenem was recorded in any of these isolates tested except non-fermentors, which suggests the absence of outer membrane porin defect in them. The 78 (77.2%) of isolates that showed susceptible to 3GC and DAT negative were true cephalosporin sensitive strains. Also, 71 (52.9%) of the AmpC negative 3GC resistance isolates may possess ESBL, which we did not tested. It was found that the sensitivity of the 3 D test is more (93.6%) when compared to Amp C Disc test (80.9%). There is an increase in sensitivity of 12.7% with the 3 D test. Amp C disc test is relatively easier to perform when compared to 3 D test. The highest percentage of AmpC-harboring isolates in clinical specimens from admitted patients thus shows its nosocomial importance. AmpC β -lactamase producing bacterial pathogens may cause a major therapeutic failure if not detected and reported in time.

Plasmid-mediated AmpC β -lactamases have been associated with false in vitro susceptibility to cephalosporins. Thus, the type of β -lactamase produced by the organism should be detected along with the antibiogram before administering the β -lactam drug to the patient. The potential benefits would include better patient outcomes in terms of avoiding inappropriate therapy. Also failure to identify AmpC β -lactamase producers may lead to inappropriate antimicrobial treatment and may result in increased mortality. This is alarming and requires urgent action from both a therapeutic and infection control perspective.

In conclusion, this study has revealed the occurrence of AmpC β -lactamase producing strains of Gram negative isolates for the first time in our region. Occurrence of a large percentage of multidrug resistant strains has been observed. AmpC β -lactamase production is frequently accompanied by multidrug resistance, thus conjugative dissemination of these AmpC β -lactamase encoding plasmids is thought to facilitate the spread of resistance against a wide range of antibiotics among different members of Enterobacteriaceae. Meropenem is superior to other antibiotics for the treatment of serious infections due to AmpC β -lactamase-producing gram-negative bacteria.

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