

## Occurrence of Plasmid-Mediated AmpC $\beta$ -Lactamases Among *Escherichia coli* and *Klebsiella pneumoniae* Clinical Isolates in a Tertiary Care Hospital in Bangalore

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**Abstract** Therapeutic options for infections caused by gram-negative organisms expressing plasmid-mediated AmpC  $\beta$ -lactamases are limited because these organisms are usually resistant to all the  $\beta$ -lactam antibiotics, except for cefepime, ceftiprome and the carbapenems. These organisms are a major concern in nosocomial infections and should therefore be monitored in surveillance studies. Hence, this study was aimed out to determine the prevalence of plasmid-mediated AmpC  $\beta$ -lactamases in *E. coli* and *K. pneumoniae* from a tertiary care in Bangalore. A total of 63 *E. coli* and 27 *K. pneumoniae* were collected from a tertiary care hospital in Bangalore from February 2008 to July 2008. The isolates with decreased susceptibility to ceftiofloxacin were subjected to confirmation test with three dimensional extract tests. Minimum inhibitory concentrations (MICs) were determined by agar dilution method. Conjugation experiments, plasmid profiling and susceptibility testing were carried out to investigate the underlying mechanism of resistance. In our study, 52 (57.7%) isolates showed resistance to ceftiofloxacin, the occurrence of AmpC was found to be 7.7% of the total isolates. Plasmid analysis of the selected isolates showed the presence of a single plasmid of 26 kb in *E. coli* and 2 Kb in *K. pneumoniae*. Plasmid-mediated AmpC  $\beta$ -lactamases were found in 11.1% of *K. pneumoniae* and in 6.3% of *E. coli*. Curing and conjugation experiments showed that resistance to cephalosporins and cephamycins was plasmid-mediated. Our study has demonstrated the occurrence of plasmid-mediated AmpC in *E. coli* and

*K. pneumoniae* which illustrates the importance of molecular surveillance in tracking AmpC-producing strains at general hospitals and emphasizes the need for epidemiological monitoring.

**Keywords** AmpC · *E. coli* · *K. pneumoniae* · Ceftiofloxacin · Antibiotic resistance · Plasmid curing

### Introduction

Bacteria over expressing AmpC  $\beta$ -lactamases are of major clinical concern because they confer resistance to a wide variety of  $\beta$ -lactams,  $\beta$ -lactam/ $\beta$ -lactamase inhibitor combinations, and monobactams, but are susceptible to fourth generation cephalosporins and carbapenems [1–3]. Constitutive over expression of AmpC  $\beta$ -lactamases in gram-negative organisms occurs either by deregulation of the *ampC* chromosomal gene or by acquisition of a transferable *ampC* gene on a plasmid or other transferable element. The transferable *ampC* gene products are commonly called plasmid-mediated AmpC  $\beta$ -lactamases [3–5]. Emergence of plasmid-mediated AmpC  $\beta$ -lactamase producing strains is well recognized [6–8]. Phenotypically bacteria resemble chromosomal class C  $\beta$ -lactamase producers in that they confer resistance to cephalosporins and cephamycins such as, ceftiofloxacin, and are not inhibited by  $\beta$ -lactam/ $\beta$ -lactamase inhibitors [9].

AmpC  $\beta$ -lactamase genes are native to the chromosomes of many gram-negative bacilli but are missing from *Klebsiella* spp. and are rarely hyper-expressed in *E. coli*. Mobilization from the genome of species typically carrying inducible/de-repressed *bla*<sub>ampC</sub> such as *Citrobacter freundii* and *Morganella morganii* by plasmids into *K. pneumoniae* and *E. coli* in which they confer novel resistance pattern [7, 8, 10, 11].

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Despite of increased interest in the recognition of plasmid-mediated AmpC  $\beta$ -lactamases in *E. coli* and *Klebsiella* spp., the worldwide distribution and prevalence of AmpC mediated resistance is fragmentary. This is due in part to the limited number of surveillance studies seeking clinical strains producing AmpC  $\beta$ -lactamases and the difficulty that laboratories have in accurately detecting these resistance mechanism(s) particularly as there are no published CLSI criteria [12]. In addition it has been stated that the detection of AmpC  $\beta$ -lactamases production is challenging since the hyperproduction of chromosomal AmpC in association with OMP F porin loss in *E. coli* or porin deficiency in *K. pneumoniae* can produce similar resistance phenotypes. Therefore, detection of plasmid-mediated AmpC enzyme producing isolates is considered critical for epidemiological studies and hospital infection control, because the genes can be spread to other organisms. Hence, the study was conducted to determine the prevalence of plasmid-mediated AmpC  $\beta$ -lactamases in clinical isolates of *Escherichia coli* and *Klebsiella Pneumoniae* and their antibiotic susceptibility pattern.

## Materials and Methods

### Clinical Strains

In a duration of six months (February 2008–July 2008), 90 consecutive non repetitive clinical isolates of *E. coli* ( $n = 63$ ) and *K. pneumoniae* ( $n = 27$ ) were obtained from Sri Bhagawan Mahaveer Jain hospital, Bangalore. The sources of isolates were from urine, throat swab, pus. Significant proportions of the strains were from the hospitalized patients (41). All isolates were identified, using the standard conventional microbiological techniques [13].

### Antibiotic Susceptibility Testing

All the bacterial isolates were tested for cefoxitin disk (30  $\mu$ g) resistance. The isolates with cefoxitin zone diameter less than 18 mm were further subjected to detailed susceptibility testing by standard disk diffusion method to note the concomitant resistance to other antibiotics. Disk diffusion tests were performed and the results were interpreted according to the guidelines of the CLSI [14]. The antibiotics used include nine  $\beta$ -lactams—ampicillin (10  $\mu$ g), aztreonam (30  $\mu$ g), ceftazidime (30  $\mu$ g), ceftriaxone (30  $\mu$ g) cefuroxime (30  $\mu$ g), cefotaxime (30  $\mu$ g), cefepime (30  $\mu$ g), imipenem, and amoxicillin/clavulanic acid (10  $\mu$ g) and three non- $\beta$ -lactam antibiotics, gentamicin (10  $\mu$ g), amikacin (30  $\mu$ g), and ciprofloxacin (5  $\mu$ g). *E. coli* ATCC 25922 was used as a quality control strain.

### MICs Determination

MICs of antimicrobial agents was carried out by the agar dilution method with Mueller–Hinton agar according to the 2005 CLSI recommendations [15], including ten  $\beta$ -lactams—ampicillin (10  $\mu$ g), aztreonam (30  $\mu$ g), ceftazidime (30  $\mu$ g), ceftriaxone (30  $\mu$ g) cefuroxime (30  $\mu$ g), cefotaxime (30  $\mu$ g), cefepime (30  $\mu$ g), cefoxitin (30  $\mu$ g), imipenem, and amoxicillin/clavulanic acid (10  $\mu$ g) and three non- $\beta$ -lactam antibiotics—gentamicin (10  $\mu$ g), amikacin (30  $\mu$ g), and ciprofloxacin (5  $\mu$ g). *E. coli* ATCC 25922 was used as a quality control strain.

### Phenotypic AmpC Detection

Since there are no published CLSI criteria for phenotypic screening or confirmatory test for AmpC  $\beta$ -lactamases, a modified three dimensional test was used for the detection of AmpC enzymes in cefoxitin resistant isolates [16].

### Plasmid Analysis and Curing Experiments

The selected AmpC producing strains of *E. coli* and *K. pneumoniae* were screened for plasmids by the alkaline lysis method [17]. Plasmid curing experiments was also performed as described by Shahid et al. [18]. Ethidium bromide was used as the curing agent. The minimum inhibitory concentration of ethidium bromide was determined for the bacterial isolates in trypticase soya broth and the highest concentration permitting the growth was used. The plasmid cured colonies were then tested for antibiotic susceptibility. Concomitant loss of antibiotic resistance was compared with the loss of plasmid content.

### Transconjugation Experiments

To determine whether the resistance was transferable, transconjugation experiments were performed on the AmpC producing isolates. Seven AmpC positive isolates were used as donors and *E. coli* K12 ( $F^-S^R lac^-$ ) resistant to streptomycin, was used as the recipient strain. Equal volumes of the donor and the recipient strain cultures in Luria–Bertani (LB) broth were mixed. The mixtures were then incubated at 37°C for 16 h. The transconjugants were selected on MacConkey agar plates supplemented with streptomycin (500  $\mu$ g/ml) and ceftazidime (2  $\mu$ g/ml) and were analyzed for plasmids as described above. Recipient *E. coli* K12 ( $F^-S^R lac^-$ ) was screened for antibiotic susceptibility by agar dilution method with reference to the guidelines of CLSI, the presence of plasmids before the conjugation, followed by similar screening of the transconjugants after mating experiments.

## Results

### Clinical Specimens and Bacterial Species Distribution

Out of the 63 isolates of *E. coli*, 52 isolates were from urine, 4 from throat swab and 7 from pus. Of the 27 *K. pneumoniae* isolates, 22 isolates were from urine, 2 from throat swab and 3 from pus. Among 63 *E. coli* and 27 *K. pneumoniae* isolates, 52 isolates (57.77%) showed cefoxitin zone diameter  $\leq 18$  mm and were considered as screen positive for AmpC production. 7 of the 52 cefoxitin resistant clinical isolates yielded positive AmpC by the three dimensional extract test. The occurrence rate of AmpC producers in *E. coli* and *K. pneumoniae* was 7.77% (7/90), including 4 in *E. coli* and 3 in *K. pneumoniae*. Out of the four isolates of *E. coli*, three (75%) were from urine samples and one (25%) was from pus sample. Among the *K. pneumoniae* isolates, one (33.3%) was from urine sample and two (66.6%) were from pus samples.

### Characteristics of Antibiotic Resistance in AmpC Producing Strains

The results of antibiotic resistance rates and patterns of cefoxitin resistant isolates ( $n = 52$ ) are shown in Tables 1 and 2. All the isolates tested in the present study for antibiotic susceptibility were multidrug resistant, the majority of isolates (61.53%) being resistant to nine or more of the 12 antibiotics tested, followed by resistance to eight drugs (17.3%), seven and six drugs (5.76%) and three drugs (11.53%). Concomitant high resistance to ampicillin, amoxicillin/clavulanic acid, ciprofloxacin, aztreonam, gentamicin, was present in 98, 86.53, 88.46, 82.69, and 48% isolates.

Out of the seven AmpC producers resistance to cefipime was noticed in three (42.85%) isolates. All of the seven AmpC producing strains were susceptible to imipenem. Table 3 shows the MIC of the seven AmpC producing isolates. These seven isolates showed very similar susceptibility profiles, characterized by elevated MICs for aztreonam, ceftazidime, cefotaxime, cefoxitin, cefuroxime while MICs of imipenem for all of them were 0.5  $\mu\text{g/ml}$ . The MICs of cefepime varied among the strain isolated.

### Plasmid Analysis and Plasmid Curing

Plasmid analysis of the isolates showed the presence of a single plasmid of  $\sim 26$  Kb in *E. coli* and 2 Kb in *K. pneumoniae*. Curing experiments were attempted in these isolates to determine the change in the plasmid content associated with the antibiotic resistance pattern. Curing experiments using the intercalating dye ethidium bromide was successful only in 85.7% (6/7) isolates. The

**Table 1** Resistance rates of other antibiotics tested in cefoxitin resistant isolates ( $n = 52$ )

Antibiotics tested	% Resistance
Aztreonam	82.69
Amoxicillin/clavulanic acid	86.53
Ampicillin	98.0
Ceftazidime	82.69
Cefotaxime	90.38
Ceftriaxone	84.61
Cefuroxime	92.30
Cefepime	67.30
Imipenem	0
Gentamicin	48.0
Amikacin	7.69
Ciprofloxacin	88.46

MICs of the ethidium bromide ranged from 400 to 600  $\mu\text{g/ml}$  for these isolates. The loss of antibiotic resistance was concomitant with the loss of plasmid content. It was noted that all the isolates that lost plasmids became susceptible to cefoxitin and gentamicin.

### Transfer of AmpC $\beta$ -Lactamases and Antimicrobial Resistance in Transconjugation

All the AmpC producing isolates subjected to transconjugation experiments were able to transfer resistance to cefoxitin and cephalosporins. The MIC of cefoxitin against the transconjugants is  $\geq 256$   $\mu\text{g/ml}$ , representing a 32-fold increase relative to that of the recipient *E. coli* K12 ( $F^-S^R lac^-$ ) strain (8  $\mu\text{g/ml}$ ). All transconjugants showed elevated MICs for ampicillin, aztreonam, ceftazidime, cefotaxime, cefoxitin, ceftriaxone and gentamicin. Additionally, MICs of the transconjugants to  $\beta$ -lactam in combination with  $\beta$ -lactamase inhibitors showed no significant synergy, which is one of the characteristics of the AmpC enzyme. The recipient strain *E. coli* K12 ( $F^-S^R lac^-$ ) did not show the presence of any plasmid before the transconjugation experimentation. The plasmid profile of the donors and transconjugants are shown in Fig. 1.

## Discussion

Plasmid-mediated AmpC  $\beta$ -lactamases have been found worldwide but are less common than extended-spectrum  $\beta$ -lactamases (ESBLs), and in *E. coli*, they appear to be less often a cause of cefoxitin resistance than an increased production of chromosomal AmpC  $\beta$ -lactamase [19 and references cited therein]. Failure to detect AmpC  $\beta$ -lactamase producing strains has contributed to their uncontrolled

**Table 2** Resistance patterns of cefoxitin-resistant isolates ( $n = 52$ )

Resistant to	Resistance patterns ( $n$ )	Total
Ten drugs	Ca Ci Ce Cpm Cu A Ac Ao Cf G (15)	17
	Ca Ci Ce Cu A Ac Ao Cf G Ak (2)	
Nine drugs	Ca Ci Ce Cpm Cu A Ac Cf G (03)	15
	Ca Ci Ce Cpm Cu A Ac Ao Cf (10)	
	Ca Ci Ce Cpm Cu A Cf G Ak (02)	
Eight drugs	Ca Ci Ce Cpm Cu A Ac Cf (03)	09
	Ca Ci Ce Cpm Cu A Ao Cf (02)	
	Ca Ci Ce Cu A Ac Cf G (01)	
	Ca Ci Ce Cu A Ac Ao Cf (02)	
	Ci Ce Cu A Ac Ao Cf G (01)	
Seven drugs	Ca Ci Ce Cu A Ac Ao (02)	03
	Ca Ci Ce A Ao Cf G (01)	
Six drugs	Ce Cu A Ac Ao Cf (03)	03
Three drugs	Cu Cf Ao (01)	05
	Cu A Ao (01)	
	A Ac Ao (03)	

Antibiotics key: *Ca* ceftazidime, *Ci* ceftriaxone, *Ce* cephotaxime, *Cu* cefuroxime, *Cpm* cefepime, *A* ampicillin, *Ao* aztreonam, *Ac* amoxicillin/clavulanic acid, *G* gentamicin, *Ak* amikacin, *Cf* ciprofloxacin

spread and therapeutic failures [20]. Hence, their appearance in hospital settings should be identified quickly so that appropriate antibiotic usage and containment measures can be implemented.

In India, AmpC producing strains of *Enterobacteriaceae* have emerged as a challenge in hospitalized as well as community based patients [21]. In 2003, 20.7% AmpC producers were found among gram-negative isolates in Guru Tegh Bahadur Hospital, Delhi [22]. In the same year, Subha et al. found AmpC  $\beta$ -lactamase production in 24.1% of *Klebsiella* spp. and 37% of *E. coli* in Chennai [23]. Around the same time, Shahid et al. reported 20 *P. aeruginosa* isolates as producing AmpC beta lactamases in Aligarh [18]. In 2005, data from Chennai revealed AmpC production in 20.8% *Klebsiella* spp. and 16.6% *E. coli* [24]. In the present study 7.77% of the isolates were found to be to be AmpC producers which were less than that reported from Delhi, Chennai and Aligarh. This difference may be due to the difference in selection criteria of isolates, the variation in the ability to produce AmpC  $\beta$ -lactamases among different gram negative bacteria and different clinical specimens. The data generated in our study revealed a relatively higher percentage of gram negative isolates producing these enzymes when compared to earlier report of 3.3% for *E. coli* and 2.2% for *K. pneumoniae* isolates in Karnataka [25], this recent increase in AmpC producing isolates may be indicative of the ominous trend of more and more isolates acquiring resistance mechanisms rendering the antimicrobial armamarium ineffective.

Of the 52 cefoxitin-resistant isolates, 45 (86.53%) were negative for AmpC  $\beta$ -lactamase production by three dimensional method. Cefoxitin resistance in these isolates could be due to the lack of permeation porins [22]. In these

strains, cefoxitin resistance can be explained by the loss of porins. Hernandez et al. demonstrated that interruption of a porin gene by insertion sequences is a common type of mutation that causes the loss of porin expression and increased cefoxitin resistance [26]. Interestingly, no AmpC harboring isolates revealed susceptibility to cefoxitin in our study or else, as we have used only cefoxitin resistance-based screening test.

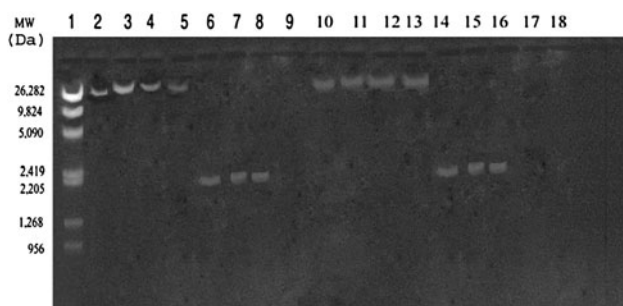
Previous studies suggested that cefepime might be effective for the treatment of infections caused by AmpC producing organisms. However, in this study, 42.85% (3 of 7) AmpC  $\beta$ -lactamase producers were resistant to cefepime. Song et al. reported that the association of plasmid-mediated AmpC  $\beta$ -lactamases with ESBLs may cause the failure of treatment and there is also a report indicating the high inoculum effect of cefepime in plasmid-mediated AmpC  $\beta$ -lactamase producing isolates [27–29]. Similar data has been published in the MYSTIC Program in Europe and the US (1997–2004) [30]. The available data suggest that carbapenems are more effective than cefepime in treating serious infections that involve large numbers of AmpC producing organisms.

In our study, imipenam was the only drug found unaffected by the action of these enzymes. These findings are in concordance with the studies conducted by other authors, who also reported a rate of 100% sensitivity to imipenam [31–35]. These carbapenam agents may be beneficial in treatment of AmpC infection; however, indiscriminate use of these agents may promote increased resistance to carbapenam.

The transconjugation study showed that resistance genes producing AmpC in seven isolates were conjugated successfully, suggesting that it is located on a transferable

**Table 3** Antibiotic resistance and MIC distributions of the seven plasmid mediated AmpC producing *E. coli* and *K. pneumoniae*

Antimicrobial agents	Resistance rate (%)	MIC distribution ( $\mu\text{g/ml}$ )
Aztreonam	85.7	>128
Amoxicillin/clavulanic acid	100	64
Ampicillin	85.7	>128
Ceftazidime	85.7	128
Cefotaxime	85.7	256
Cefoxitin	100	256
Ceftriaxone	71.42	>64
Cefuroxime	85.7	>128
Cefepime	42.9	64(1), 32 (1), 8(1)
Imipenem	0	0.5
Gentamicin	28.6	128
Amikacin	14.2	4
Ciprofloxacin	71.4	64



**Fig. 1** Agarose gel (0.8%) showing 26 and 2 Kb plasmids in donors (Lane 2–8) and their respective transconjugants (Lane 10–16). Molecular weight marker  $\lambda$ /Mlu Hind digest along with their size (in Daltons) is shown in lane 1. The recipient strain (*E. coli* K12F<sup>-</sup>S<sup>R</sup> lac<sup>-</sup>) screened for plasmids before transconjugation experiment was found devoid of the same (lane 9)

element such as a conjugative plasmid. The transfer of plasmids encoding  $\beta$ -lactamase genes into *E. coli* K12 (F<sup>-</sup>S<sup>R</sup> lac<sup>-</sup>) was accompanied by resistance to cephalosporins as well as decreased susceptibility to gentamicin. Thus it indicated that the resistance to  $\beta$ -lactams and aminoglycosides coexisted.

Plasmid analysis of the representative isolates showed the consistent presence of a single plasmid. Successfully cured isolates became susceptible to cephamycins and cephalosporins thus providing evidence of carriage of *bla*<sub>ampC</sub> genes on the plasmid. Dissemination of these AmpC  $\beta$ -lactamase encoding plasmids is thought to facilitate the spread of resistance against a wide range of antibiotics among *K. pneumoniae* and *E. coli*.

Cephalosporin resistance among *K. pneumoniae* and *E. coli* has increased worldwide [36] as shown in the present antimicrobial susceptibility data, but the rates of resistance to cephalosporins, including that to cefepime, are high. However, all AmpC producing isolates remain susceptible to imipenem. Multi-resistant organisms should

be treated with antibiotic regimens other than cephalosporins. Continuous or frequent use of cephalosporins probably leads to higher resistance rates of AmpC producing isolates of *Enterobacteriaceae*.

Therefore, it would be wise to perform surveillance of clinical isolates to monitor resistance levels in the different wards [37]. In addition, present findings may have important implications in the control of AmpC  $\beta$ -lactamase-producing *K. pneumoniae* and *E. coli* strains, which are likely to be overlooked in hospitals. In order to prevent the spread of resistant hospital flora, we suggest restriction of the prescription of broad-spectrum antimicrobial agents is necessary [38].

This is a preliminary study designed with an objective to detect the possible occurrence of AmpC  $\beta$ -lactamases in a tertiary care hospital and to institute antibiotic policy to minimize the emergence of antimicrobial resistance. This is perhaps the first report of Plasmid-mediated AmpC  $\beta$ -lactamase production among gram-negative clinical isolates from this city.

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