Occurrence of \( \text{bla}_{\text{ampC}} \) in cefoxitin-resistant \textit{Escherichia coli} and \textit{Klebsiella pneumoniae} isolates from a North Indian tertiary care hospital

Farrukh Sobia, Mohammad Shahid, Anuradha Singh, Haris M. Khan, Indu Shukla and Abida Malik

Abstract

Aim: To analyse the occurrence of \( \text{bla}_{\text{ampC}} \) in cefoxitin-resistant isolates and to evaluate the role of phenotypic methods for detection of AmpC-producers.

Methods: A total of 91 isolates (84 \textit{E. coli} and 7 \textit{K. pneumoniae}) that were resistant to cefoxitin and obtained during a period of six months were studied. Antibiotic susceptibility to third- and fourth-generation cephalosporins and other antibiotics were performed. \( \text{bla}_{\text{ampC}} \) was detected by PCR.

Results: Cefoxitin-resistant isolates showed concomitant resistance to other antibiotics used. All 91 isolates showed multiple antimicrobial resistance and were found resistant to more than three antibiotics; maximum resistance (among cephalosporins) was noticed for ceftriaxone (89%) followed by cefpirome (80%). A total of 82.4% isolates were found positive for \( \text{ampC} \) gene, however, 57.1% were found AmpC-producers by modified three-dimensional extract test (MTDET). Out of 57.1% isolates that were noticed as AmpC-producers by MTDET, 50.5% were found positive on detection by PCR but 6 (6.6%) isolates were found negative for presence of \( \text{ampC} \) gene. Among 26 (28.6%) AmpC-intermediate isolates, 20 (21.9%) were found to harbor \( \text{ampC} \) genes while 6 (6.6%) were found negative for the same. Of the 14.3% isolates that were considered negative by TDET, 10.9% gave positive results by PCR. Almost a similar frequency of occurrence of cefoxitin-resistant isolates harbouring \( \text{bla}_{\text{ampC}} \) was noticed from different hospital wards.

Conclusion: Among cefoxitin-resistant isolates, prevalence of \( \text{bla}_{\text{ampC}} \) is quite high and a phenotypic test is insufficient to diagnose AmpC-producers and hence genotypic test, like PCR, should be used.

Key words: \( \text{bla}_{\text{ampC}}, \) cefoxitin resistance, TDET, PCR, \textit{E. coli}, \textit{K. pneumoniae}

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Introduction

The worldwide use of antimicrobials has created enormous pressure for the selection of resistance among opportunistic bacterial pathogen. The resistance mechanism in bacteria to \( \beta \)-lactams is the production of \( \beta \)-lactamases that catalyze the hydrolysis of the \( \beta \)-lactam ring, preventing their interaction with the D,D-transpeptidases (1). During treatment with \( \beta \)-lactams, resistant mutants showing constitutive high levels of AmpC production are frequently selected leading to therapeutic failures (2). Bacteria over-expressing AmpC \( \beta \)-lactamase are of major clinical concern as they confer resistance to beta-lactams, beta-lactam-beta-lactamase inhibitor combination and monobactams, but are found susceptible to fourth-generation cephalosporins (4GC) and carbapenems (3). Hence these two classes of drugs remain the only therapeutic options for such organisms (4,5). However, isolates harboring extended-spectrum beta-lactamases (ESBLs) along with AmpC offers resistance to 4GC as well (6).

The increasing awareness and improved recognition of ESBL-producers have led to improved infection control measures to minimize spread of these emerging pathogens. With no published CLSI (formerly NCCLS) guidelines for proper identification and infection control measures, AmpC producing organisms infections may become a greater concern than ESBL producing organisms infections as they are increasing in prevalence (7,8). In addition MDR plasmids harboring both ESBL and AmpC genes are spreading among bacteria and are becoming a new emerging threat.

AmpC beta-lactamase poses a serious risk of transmission to hospitalized patients when colonized or infected ones are admitted, presenting a concern for hospital infection control surveillance of these resistance mechanisms. In the present study we analysed the occurrence of \( \text{bla}_{\text{ampC}} \) in a collection of cefoxitin-resistant isolates and evaluated the role of phenotypic methods for detection of AmpC-producers.

Methods

A total of 91 isolates (84 \textit{E. coli} and 7 \textit{K. pneumoniae}) were obtained from 91 hospitalised patients admitted to the Jawaharlal Nehru Medical College & Hospital over a period of six months (January to June 2009) for the study. Demographic details of the patients were also noted. All these isolates were found to be resistant to cefoxitin and were isolated from urine, pus, semen, cervical swabs, drains and CSF.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed and interpreted as per CLSI guidelines (9). The following antibiotics (all supplied from Hi Media Laboratories, Mumbai, India) were tested: ceftriaxone (30μg), cefoperazone (75μg), cefixime (30μg), cefazidime (30μg), cephalosporin (30μg), cefpirome (30μg), gentamicin (10μg), amikacin (30μg), ofloxacin (5μg), gatifloxacin (5μg) and aztreonam (30μg).

Phenotypic detection of AmpC-producers

Modified three-dimensional extract test (MTDET), as described by Shahid et al.(10) was performed on all cefoxitin-resistant isolates to identify AmpC producers among them. Briefly, 10-15 mg of bacterial wet weight was scraped from the culture plate and suspended in 0.5mL of peptone water in a sterile micro-centrifuge tube and incubated at 37°C for one hour. Crude enzyme extract was prepared by repeated freezing-thawing. To ensure complete membrane lysis, the freezing-thawing was carried out five times. Lawn culture of \textit{E. coli} ATCC 25922 was prepared on Mueller-Hinton Agar (MHA) plate and was incubated at 37°C for one hour. Crude enzyme extract was prepared by repeated freezing-thawing. To ensure complete membrane lysis, the freezing-thawing was carried out five times. Lawn culture of \textit{E. coli} ATCC 25922 was prepared on Mueller-Hinton Agar (MHA) plate and was incubated at 37°C for one hour. Crude enzyme extract was prepared by repeated freezing-thawing. To ensure complete membrane lysis, the freezing-thawing was carried out five times.
**Genotypic detection of bla**<sub>ampC</sub> genes**

*bla*<sub>ampC</sub> genes were detected by PCR as described by Feria et al.(11) with some modifications. Briefly, the gene of interest was amplified in a total reaction volume of 50μl containing 10pmol each of primer (ampC-f, 5’CCC CGC TTA TAG AGC AAC AA 3’ and ampC-r, 5’TCA ATG GTC GAC TTC ACA CC 3’) that span universal region of *ampC* gene, 0.2mM of each dNTPs, 10mM Tris-HCl (pH 8.8), 50mM KCl, 2.0 mM MgCl<sub>2</sub> and 1.25U Taq DNA Polymerase (Bangalore Genei, India). 2μl of template DNA was added to 48 μl of master mixture. The reaction mixture was placed in MJ-mini Bio-Rad thermal cycler (Bio-Rad, USA). The PCR amplification cycle was performed with cycling conditions consisting of an initial denaturation step at 95˚C for 15 min, followed by 35 cycles of 94˚C for 60 sec., 58˚C for 2 min., 72˚C for 3 min. and the process was completed with a final elongation step at 72˚C for 10 min. Amplified PCR products were analysed by gel electrophoresis with 2% agarose (Bangalore Genei, India) gel containing ethidium bromide. After electrophoresis DNA fragments were visualized by Bio-Rad Gel documentation system (Bio-Rad, USA).

**RAPD typing**

Genotyping of cefoxitin-resistant isolates was done as described previously (12) in order to determine whether any specific clone was circulating in our hospital environment. The results were analyzed by using Bio-Rad Gel documentation system (Bio-Rad, USA).

**Plasmid analysis**

Plasmid isolation was done in all isolates by the large scale alkaline lysis method as described previously (13). 10 μL of plasmid samples were electrophoresed in 0.8% agarose gel containing ethidium bromide. A DNA double-digested with EcoRI and HindIII (Bangalore Genei, India) was used as a molecular weight marker.

**Results and Discussion**

AmpC producing strains which are intrinsically resistant to clavulanic acid are causing great concern as carbapenems are the only antibiotics effective against such strains (14). With the spread of these strains all over the world it is necessary to know their prevalence in a hospital so as to formulate a policy of empirical therapy in high risk units. It becomes equally important that information should be procured on an isolate from a patient so as to avoid misuse of extended spectrum cephalosporins. The routine susceptibility tests performed by clinical laboratories fail to detect these strains, which may lead to inappropriate and unsuccessful therapy of the patient and unnecessary usage of drugs (15).

On analyzing the clinical and demographic features (Table 1) in the present study it was observed that the mean ages of patients having infection of cefoxitin-resistant *E. coli* and *K. pneumoniae* were 30.4 and 37.7 years, respectively. The source of cefoxitin-resistant *E. coli* was pus (50.0 %), urine (38.1 %), drain (6.0 %), semen (3.6 %), and 1.2 % in both cervical swab and CSF, while 85.2 % *K. pneumoniae* were obtained from pus and 14.3% from urine. Maximum number of isolates were obtained from the surgery ward (33 *E. coli* and 4 *K. pneumoniae*) followed by the gynaecology and orthopaedics wards (19 *E. coli* and 2 *K. pneumoniae* & 17 *E. coli* and 1 *K. pneumoniae* respectively). Cefoxitin-resistance can be used to screen isolates for detecting possible AmpC production, but lack of permeation of porins has also been reported as one of the resistance mechanism of cefoxitin in AmpC non-producers (15).

All test isolates were subjected to antimicrobial susceptibility testing and it was observed that maximum resistance (among cephalosporins) was encountered for ceftriaxone (89%) followed by cefpirome (80%), but the highest resistance rate was observed for aztreonam (91%). A fluoroquinolone, ofloxacin, also represented a quite high resistance rate (90%). Least resistance was observed for amikacin (26%) followed by gentamicin, indicating that resistance to aminoglycosides is not prevailing in our environment. Details of antimicrobial resistance pattern of the tested isolates are shown in Figure 1.

Unlike ESBLs, the detection method of AmpC β-lactamase has not been standardized by CLSI and hence it is a major barrier in defining the actual prevalence and epidemiology of these β-lactamases. The isolates producing this group of β-lactamase are typically designated as ESBL-negative and would not be tested further or sometimes they are categorized as ESBL-producers (16). Hence, in the present study, the cefoxitin-resistant isolates were subjected to MTDET for phenotypic confirmation of AmpC-producers and the results were analysed against genotypic (PCR) test to evaluate the role of phenotypic test (which is being used in most of clinical parameters) for detection of AmpC-producers. Three types of results were observed in MTDET viz. 57.1 % isolates showed maximal distortion of zone of inhibition and hence labeled as AmpC-producers, 14.3 % isolates showed no distortion and were categorized as AmpC non-producers and 28.6 % isolates showed minimal distortion and were interpreted as AmpC-intermediate (Figure 2). Among AmpC producers, 47 were *E. coli* and 5 were *K. pneumoniae*. Similarly, among AmpC non-producers, 13 were *E. coli* but none of the *Klebsiella* was interpreted as AmpC non-producer. However, 24 *E. coli* & 2 *Klebsiella* were grouped in the AmpC-intermediate category.

![Figure 1. Antibiotic resistance profile of the clinical isolates studied.](Image 305x116 to 567x341)

**Figure 1.**

Antibiotic resistance profile of the clinical isolates studied.

![Figure 2. Isolate A showing maximum distortion of zone of inhibition (AmpC-producer), B indicating minimal distortion (AmpC-intermediate type) and C indicates no distortion (AmpC non-producers). PC is positive control.](Image 305x587 to 567x767)
These isolates were subjected to genotypic detection of \( \text{ampC} \) gene to know the exact prevalence of AmpC-producers and to evaluate the exact role of phenotypic test like TDET in AmpC-detection. A total of 82.4 % isolates were found positive for \( \text{ampC} \) gene (Figure 3). Out of 57.1% isolates that were labeled as AmpC-producers by phenotypic detection method (TDET), 50.5 % were found positive on detection by PCR but 6 (6.6 %) isolates were found negative for presence of \( \text{bla}\_{\text{ampC}} \) gene. Among 26 (28.6%) AmpC-intermediate isolates, 20 (21.9%) were found to harbor \( \text{bla}\_{\text{ampC}} \) genes while 6 (6.6%) were found negative for the same. However, 14.3 % isolates were found negative by TDET but among these, 10.9 % gave a positive result on genotypic detection. Hence, a significant variation was observed in phenotypic and genotypic results and a significant number (9/91) of isolates harbors \( \text{bla}\_{\text{ampC}} \) could not be detected by TDET. Probably some other mechanism is playing a part or some other enzyme resembling AmpC is produced by the isolates which inactivates the cefoxitin.

Based on the reported phenotypic studies, it can be observed that there appears to be an increase in frequency of AmpC beta-lactamases as 59.4% have been reported in 2010 (24) as compared to 37.5% in 2003 (17). We have also observed an alarming rise in the prevalence of \( \text{bla}\_{\text{ampC}} \) gene in the present study as compared to our previous reports.

RAPD typing of the tested isolates have demonstrated diversity in our bacterial population. On analyzing banding patterns, 64 clusters were observed, each giving its unique banding pattern. However, few bacterial isolates from the gynaecology and orthopaedics wards displayed a similar banding pattern. It can be concluded that probably the same clone is circulating in the gynaecology, surgery and orthopaedics wards as these are sharing the same block in our hospital building and therefore the chance of cross contamination is high.

All the 91 isolates were tested for the presence of plasmid and it was observed that there occurred a consistent presence of a single plasmid of ~ 23 kb (Figure 4). This finding was similar to that observed in our previous studies (6,13). Isolates showing the presence of \( \text{bla}\_{\text{ampC}} \) genes also showed the presence of plasmid, except two isolates, where we obtained amplified product for the \( \text{bla}\_{\text{ampC}} \) gene, but the plasmid was absent. These results indicate that \( \text{bla}\_{\text{ampC}} \) gene is also present on chromosomes of a small proportion of the bacterial population.

![Figure 3](image3.png)

**Figure 3.** Agarose gel (2.0%) showing results of PCR for detection of \( \text{bla}\_{\text{ampC}} \) genes. Molecular weight markers (High range DNA Ladder, Bangalore Genei, India) along with their sizes (in bp) are shown in Lane 1 & 15. Lane 2 shows positive control strain (Citrobacter D1) for \( \text{bla}_{\text{ampC}} \) gene. Lane 3 shows negative control with no DNA template. Lane 4-11 shows \( \text{bla}_{\text{ampC}} \) amplicons (634 bp) from clinical isolates while lane 12-14 shows negative clinical samples.

Out of 37 isolates obtained from the surgery ward, 31 (83.8%) showed the presence of \( \text{bla}\_{\text{ampC}} \) genes. Similarly, 81.0% (17/21) and 72.2% (13/18) occurrence of \( \text{bla}\_{\text{ampC}} \) genes were from the gynaecology and orthopaedics wards respectively (Table 1 for detailed results). These results indicate that \( \text{bla}\_{\text{ampC}} \) harboring isolates are in uniform circulation in our hospital environment.

Although reported with increasing frequency, the actual prevalence of AmpC beta-lactamase is still unknown as few studies have examined frequency of this class of beta-lactamase and they too have been described on the basis of phenotypic detection methods only. In India, 37.5 % and 47.8 % AmpC-producers have been reported from Chennai and Kolkata respectively (17,18). A total of 8 % isolates were reported as AmpC-producers by Singh et al.(2). Moreover, they have reported 36% of cefoxitin-resistant isolates as AmpC-producers which were confirmed by three Dimensional Extract test and also by AmpC disc test. They have categorized these phenotypically characterized isolates as strong (24.6%) and weak (11.5%) AmpC-producers by AmpC disc test, while 43 % AmpC-producers were reported by Manchanda & Singh (19). Hemlatha et al.(20) from Chennai observed 47.3 % AmpC producers in \( E.\ coli \) and \( Klebsiella \) isolates by an inhibitor-based method using boronic acid. Recently, Sinha et al.(21) have reported 24.0 % AmpC-producers in \( E.\ coli \) isolated from a tertiary care hospital in Jaipur and observed 27.5 % of AmpC non-producer isolates as cefoxitin-resistant. In a study conducted in Pondicherry, South India, 80.9% (51/63) isolates were described as AmpC-producers by AmpC disc method and 93.6% (59/63) by three-dimensional extract test method (22).

Tan et al.(23) have reported AmpC activity in 49.8% isolates based on phenotypic detection methods, while they observed \( \text{bla}_{\text{ampC}} \) in 47% isolates on PCR detection. Shahid et al.(6) reported 39.1% (18/46) \( Enterobacteriaceae \) isolates harbouring \( \text{bla}_{\text{ampC}} \) from Aligarh between 2003 and 2005. Recently, Upadhyay et al.(24) reported 59.4% isolates as AmpC-producers based on phenotypic test from Varanasi, though that study did not test for the \( \text{bla}_{\text{ampC}} \) gene.

![Figure 4](image4.png)

**Figure 4.** Agarose gel (0.8 %) showing ~ 23 kb plasmids (Lanes 2-8) isolated from clinical samples. Lane 1 shows molecular weight marker (Lambda DNA double digested with Hind III and EcoRI, Bangalore Genei, India) along with their sizes (in kilo base pairs). Early identification of these organisms is necessary as the appropriate treatment might reduce the spread of these resistant strains and consequently mortality in hospitalized patients can be reduced. This emphasizes the need for the detection of isolates that produce such enzymes and hence therapeutic failures and
nosocomial outbreaks can be avoided. It can be concluded that among cefoxitin-resistant isolates, prevalence of \( \text{bla}_{\text{ampC}} \) is quite high in our region. We emphasize here that a genotypic test, like PCR, should be used for detection of AmpC-producers as a fair number of the isolates harboring \( \text{bla}_{\text{ampC}} \) could not be detected by TDET. Since few isolates found positive by TDET were not harboring \( \text{bla}_{\text{ampC}} \), we presume some other mechanism exists for these isolates. Hence, we also suggest that, to understand the exact mechanism, a combination of phenotypic and genotypic method should be used.

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Author information
Farrukh Sobia, MSc, PhD Scholar
Mohammad Shahid, MBBS MD PhD MNZIMLS, Associate Professor
Anuradha Singh, MSc, PhD Scholar
Haris M. Khan, MBBS MD, Professor
Indu Shukla, MBBS MD, Professor
Abida Malik, MBBS MD, Professor

Section of Antimicrobial Resistance Research & Molecular Biology, Department of Medical Microbiology, Jawaharlal Nehru Medical College & Hospital, Aligarh Muslim University, Aligarh, India.

Author contributions
Farrukh Sobia and Anuradha Singh collected data, conducted experiments and substantively drafted the article. Haris Khan, Indu Shukla and Abida Malik advised on method evaluation and contributed to writing the article. Mohammad Shahid conceived the study, collected data, conducted experiments and substantively drafted the article. The authors declare no conflicts of interest.

Corresponding author
Associate Professor Mohammad Shahid, Department of Medical Microbiology, Jawaharlal Nehru Medical College & Hospital, Aligarh Muslim University, Aligarh 202 002, India. E-mail: shahidsahar@yahoo.co.in

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