

## Short communication

**Detection of plasmid-mediated AmpC- $\beta$ -lactamases among clinical isolates: A diagnostic and therapeutic challenge**Ronni Mol P<sup>1,2</sup>, Ganesan Shanthi<sup>1</sup>, Amer Almarabheh<sup>3</sup>, Khalid M. Bindayna<sup>2</sup><sup>1</sup>Division of Microbiology, Rajah Muthaiah Medical College, Annamalai University, Chidambaram, Tamil Nadu, India<sup>2</sup>Department of Microbiology, Immunology and Infectious diseases, College of Medicine and Medical Sciences, Arabian Gulf University, Manama, Kingdom of Bahrain<sup>3</sup>Department of Community and Family Medicine, College of Medicine and Medical Sciences, Arabian Gulf University, Manama, Kingdom of Bahrain

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Corresponding author: **Ronni Mol P.** Email: [ronnimj@agu.edu.bh](mailto:ronnimj@agu.edu.bh)**ABSTRACT**

**Introduction and Aim:** The AmpC enzymes are cephalosporinases that impart resistance to a wide range of  $\beta$ -lactam,  $\beta$ -lactam/ $\beta$ -lactamase inhibitor combinations, and monobactams, but are sensitive to fourth generation cephalosporins and carbapenems. Identification techniques for AmpC beta lactamases are not yet adapted for the clinical laboratory, which is likely to underestimate this resistance mechanism. Detection and determination of the magnitude of AmpC is therefore critical for successful treatment and for the prevention and control of these resistant bacteria. The present study was intended to determine the prevalence of plasmid mediated AmpC genotypes among clinical isolates at a tertiary care hospital of South India.

**Materials and Methods:** It was a cross-sectional study. 94 isolates [*E. coli* (n=31) and *K. pneumoniae* (n=63)] were recovered between January 2020 and June 2020. Samples underwent an initial cefoxitin screening test and a subsequent genotypic study with multiplex polymerase chain reactions for AmpC subtypes. Antimicrobial susceptibility characteristics of these clinical isolates have also been investigated.

**Results:** Thirty-seven clinical isolates were cefoxitin-resistant and genotypic analysis showed that 22 cefoxitin-resistant isolates are AmpC positive, respectively. These AmpC producers are multidrug-resistant and *Klebsiella pneumoniae* is the dominant strain among them. Among them single AmpC production mechanism included blaDHA producers (n=5), blaCIT producers (n=4), blaEBC producers (n=3) and blaFOX producers (n=1) and 9 isolates showed multiple AmpC genes.

**Conclusion:** AmpC isolates emergence is worrisome and emphasizes the need for further surveillance in this region. It is proposed that hospitals improve the surveillance of AmpC  $\beta$ -lactamase in clinical isolates and suggest using carbapenems to treat infections caused by AmpC-producing bacteria.

**Keywords:** AmpC  $\beta$ -lactamases; Cefoxitin; *Escherichia coli*; *Klebsiella pneumoniae*; multiplex polymerase chain reaction; multidrug-resistant.

**INTRODUCTION**

The prevalence of multidrug-resistant Gram-negative bacteria has progressively increased over the last few decades, and the strains developing AmpC  $\beta$ -lactamases and/or extended spectrum  $\beta$ -lactamases (ESBLs) are of specific concern. The AmpC enzymes are cephalosporinases that impart resistance to a wide range of  $\beta$ -lactam,  $\beta$ -lactam/ $\beta$ -lactamase inhibitor combinations, and monobactams, but are sensitive to fourth generation cephalosporins and carbapenems (1). To date, the following AmpC families have been identified worldwide: CMY-1 and CMY-2 families of CMY  $\beta$ -lactamases isolated from *Aeromonas hydrophila* and *Citrobacter freundii*, respectively, FOX-type and MOX-type enzymes from *Aeromonas spp.*, the ACC family enzymes from *H. Alvei*, the MIR and ACT families derived from *Enterobacter spp.*, Cephalosporinases family from *Citrobacter Freundii*;

and the DHA- type enzymes isolated from *Morganella morganii* (2).

AmpC isolates have been frequently seen in patients who have undergone extended ICU hospitalizations, surgical operations, or who have been immunocompromised or who have had chronic illness, such as leukemia. Therapeutic options for infections caused by the AmpC producers are limited. In general, ampC-enzyme-producing bacteria cause treatment difficulties and delays in treatment, including morbidity and mortality (3). Detection and determination of the magnitude of AmpC is therefore critical for successful treatment and for the prevention and control of these resistant bacteria (4).

To our knowledge, there are limited studies related to the identification and characterization of AmpC  $\beta$ -lactamases enzymes in Enterobacteriaceae in this region. The present study was therefore intended to determine the magnitude of AmpC strains provided by clinical specimens at tertiary care hospital.

## MATERIALS AND METHODS

### Strain source

A total of 94 non-repetitive clinical isolates of *E. coli* and *K. pneumoniae* were obtained from pus and sputum samples between January 2020 and June 2020. The present study was performed in compliance with the Helsinki Declaration and was accepted by the University Ethics Committee.

### Identification of the bacteria

All the isolates *E. coli* (n=31) and *K. pneumoniae* (n=63) were identified by conventional methods as defined by Monica Cheesebrough (5) and antibiotic susceptibility was performed by Kirby Bauer method as per CLSI (Clinical and Laboratory Standard Institute, formerly NCCLS) guidelines 2019 (6).

### Screening for AmpC β-Lactamase-producing strains

Strains were screened using Kirby-Bauer diffusion test in which cefoxitin (30 µg; HIMEDIA) was used. According to the CLSI Antimicrobial Susceptibility Testing (AST) Standards, isolates with an inhibitory zone diameter of ≤18 mm were suspected to be AmpC β-lactamase producers (7).

**Table 1:** Primers used for characterization of AmpC β-lactamases (9)

Primer	Expected amplicon size (bp)	Sequence (5' to 3')
MOXMF	520	GCT GCT CAA GGA GCA CAG GAT
MOXMR		CAC ATT GAC ATA GGT GTG GTG C
CITMF	462	TGG CCA GAA CTG ACA GGC AAA
CITMR		TTT CTC CTG AAC GTG GCT GGC
DHAMF	405	AAC TTT CAC AGG TGT GCT GGG T
DHAMR		CCG TAC GCA TAC TGG CTT TGC
ACCMF	346	AAC AGC CTC AGC AGC CGG TTA
ACCMR		TTC GCC GCA ATC ATC CCT AGC
EBCMF	302	TCG GTA AAG CCG ATG TTG CGG
EBCMR		CTT CCA CTG CGG CTG CCA GTT
FOXMF	190	AAC ATG GGG TAT CAG GGA GAT G
FOXMR		CAA AGC GCG TAA CCG GAT TGG

**Statistical analysis:** Statistical Package for the Social Science (SPSS) version 20 (IBM, Armonk, NY, United States of America) were used to obtain descriptive data. The significance was calculated by Chi-Square test.

## RESULTS

### *Klebsiella pneumoniae* represents the dominant AmpC-β-lactamase-positive bacterial strain among the clinical isolates

Among the 94 isolates screened, 37(39%) isolates were recorded cefoxitin resistant including 27 samples from pus and 10 from sputum. Among the cefoxitin resistant, 15(40.5%) were *E. coli* and 22(59.4%) were *K. pneumoniae*. Multiplex PCR identified AmpC production among 22(59.4%) cefoxitin resistant isolates. Genotypic ampC-β-lactamase producers were obtained from pus (n= 15) and sputum (n= 7) and this may be due to prior antimicrobial exposure or

### Molecular characterization of AmpC resistance strains

Samples for genotypic confirmation was obtained from pus and sputum. Multiplex polymerase chain reactions (PCR) were used to detect the most common plasmid mediated AmpC genes shown in Table 1. DNA extraction was performed using the Modified Proteinase K method (8). For PCR assays, 2µl of cDNA was applied to the 23µl master mixture of PCR reagents (Qiagen Multiplex PCR Kit). The reaction was as follows: initial denaturation at 94°C for 3 minutes, followed by 25 cycles of DNA denaturation at 94°C for 30 seconds, then primer denaturation for 30 seconds at 64°C, primer extension for 1 minute at 72°C; and a final extension step at 72°C for 7 minutes (9). Amplified products were electrophoresed by 3 per cent Agarose gel containing 1x TAE (Tris Acetate EDTA buffer) and 16 µl of each amplified product was loaded into each well. Electrophoresis was conducted at 25V for 2 hours. After staining with ethidium bromide (0.5µg/ml), the gel was visualized under UV light illuminator. The gel image has been captured and analyzed using the Gel Documentation Method (Major Science, USA).

health-related acquisition in patients. 38% of cefoxitin-resistant isolates contain *Klebsiella pneumoniae* strain (n=14) while the remaining samples are *Escherichia coli* strain (n=8). Among them single ampC production mechanism included 22.70% *bla<sub>DHA</sub>* producers (n=5), 18.18% *bla<sub>CIT</sub>* producers (n=4), 13.60% *bla<sub>EBC</sub>* producers (n=3) and 4.54% *bla<sub>FOX</sub>* producers (n=1). Nine isolates showed multiple ampC production as shown in Table 2.

**Table 2:** Multiple AmpC producing isolates

Multiple AmpC mechanisms	Number of strains (n=9)
ACC + CIT	1
ACC + DHA	1
ACC + MOX	1
FOX + CIT	1
FOX + ACC + CIT	2
FOX + DHA + CIT	1
FOX + DHA + CIT + MOX	1
FOX + ACC + CIT + MOX	1

### Multidrug resistance is a cardinal characteristic of cefoxitin-resistant and genotypic AmpC-positive isolates.

Drug resistance research has been conducted for many types of antibiotics including amikacin, gentamicin, cefuroxime, piperacillin/tazobactam, ciprofloxacin, cotrimoxazole, meropenem as detailed in Table 3 and Table 4.

Most of the clinical isolates showed resistance to amikacin, gentamicin, cefuroxime, ciprofloxacin and cotrimoxazole except for piperacillin and tazobactam and meropenem. Interestingly, the incidence of genotypically ampC-positive isolates that are resistant to ciprofloxacin and cotrimoxazole is substantially higher than that of cefoxitin resistant and AmpC-negative isolates (Table 3 and Table 4).

**Table 3:** Antibiotic resistance pattern of Cefoxitin resistant and AmpC negative strains

Antibiotic Type	Cefoxitin resistant strains (n=37) no. (%)	AmpC negative strains (n=72) no. (%)	95% C. I.	P value
Amikacin	4 (11)	19 (26)	-0.299 - -0.013	0.032*
Gentamicin	7 (19)	33 (46)	-0.440 - -0.098	0.002*
Cefuroxime	27 (73)	55 (76)	-0.208 - 0.139	0.700
Piperacillin/Tazobactam	0 (0)	2 (2.7)	-0.066 - 0.010	0.151
Ciprofloxacin	17 (46)	33 (46)	-0.196 - 0.199	0.991
Cotrimoxazole	20 (54)	45 (62.5)	-0.280 - 0.111	0.398
Meropenem	0 (0)	0 (0)		

\* P-value < 0.05 is considered statistically significant.

**Table 4:** Antibiotic resistance pattern of AmpC positive and AmpC negative strains

Antibiotic Type	AmpC positive strains (n=22) no. (%)	AmpC negative strains (n=72) no. (%)	95% C. I.	P-value
Amikacin	2 (9)	19 (26)	-0.330 - 0.016	0.031*
Gentamicin	5 (23)	33 (46)	-0.441 - -0.022	0.031*
Cefuroxime	17 (77)	55 (76)	-0.192 - 0.210	0.931
Piperacillin/Tazobactam	0 (0)	2 (2.7)	-0.066 - 0.010	0.151
Ciprofloxacin	14 (64)	33 (46)	-0.054 - 0.410	0.132
Cotrimoxazole	15 (68)	45 (62.5)	-0.168 - 0.281	0.620
Meropenem	0 (0)	0 (0)		

\* P-value < 0.05 is considered statistically significant.

In addition, while multiple drug resistance has been noted in both genotypically ampC-positive isolates and ampC-negative isolates, the proportion of genotypically ampC-positive isolates (59%) that are resistant to three or more antibiotics is more than that of ampC-negative isolates (19.4%). Collectively, these findings are consistent with the idea that multidrug resistance in clinical isolates is correlated with the development of ampC β-lactamase.

### DISCUSSION

Concerns about antibiotic susceptibility against ampC producers can complicate therapeutic decisions. Almost 250 distinct ampC β-lactamases with different geographical distributions have been identified (10). While there are no CLSI guidelines for ampC detection currently, resistance to cefoxitin has been used as a predictor for ampC clinical isolates. In the present analysis, 39% of all the clinical isolates are resistant to cefoxitin. Cefoxitin resistance can also be mediated by alterations of the outer membrane permeability (11). A high level of cefoxitin resistance (59%) was reported from India by Handa *et al.*, (12). Another study reported 5.4% of all the clinical isolates resistant to cefoxitin (3). In several studies, including the present study, there were small proportion of non-

AmpC producers among cefoxitin resistant isolates (3, 12). Resistance in these isolates may be due to a change in cell permeability to cefoxitin caused by porin loss or the involvement of other beta-lactamases (13).

Infectious disease expert studies in Singapore, Australia, and New Zealand found that more than half of clinicians chose to treat suspected AmpC producing infections with carbapenem (58%), remainder using either cefepime (19%) or piperacillin/tazobactam (8%; 14). In this research, all ampC positive isolates were susceptible to meropenem and piperacillin/tazobactam, which concludes that these antibiotics can be used as the preferred medication for the treatment of ampC producing infections. A study by Mohamudha *et al.* also found no resistance to carbapenems among the isolates tested (15).

Plasmid-mediated AmpC β-lactamases are widespread among Enterobacteriaceae in India with 66% of *E. Coli* and 35 % of the *Klebsiella* species as AmpC producers (10). However other reports from India showed a range of prevalence from 2.2% to 20.7% (3). AmpC production was reported in this study as 37.8 % of the *Klebsiella* species and 21.6 % of *E. coli*, are isolates that were resistant to cefoxitin,

indicating a similar development rate of AmpC β-lactamase in our region also.

In terms of diagnosis, knowledge of molecular subtypes and the prevalence of plasmid derived AmpC in various geographical areas is critically essential for proper consideration of antimicrobial therapy and successful control of infections. The worldwide prevalence of plasmid mediated Amp C ranged widely from 2% to 46% (16). In Indian studies, the prevalence of Amp C ranged from 8 to 47%. Overall, present study reported a prevalence of 23.4% AmpC producers through PCR analysis. A study by Shanthi *et al.*, also observed a prevalence of 29.8% (n=23) AmpC producers from the study isolates (13).

In view of the limitations of the various phenotypic assays, molecular methods are considered to be the gold standard for the detection of AmpC β-lactamases (10). In this research, the multiplex PCR assay classified 22 isolates as AmpC producers (59.4%), with the most common AmpC mechanism being blaDHA (22.70%), followed by blaCIT (18.18%) and blaEBC (13.60%), and blaFOX (4.54%) which was not consistent with other literatures (10). Another study from Southern India reported CIT family (CMY-2 to CMY-7, LAT-1 to LAT-4 and BIL-1) to be most common, followed by DHA and EBC (13). Aside from the geographical diversity of AmpC strains, the most common subtypes of AmpC producers in North Africa and Australia have been identified as CMY, DHA, and EBC (17). The presence of multiple AmpC genes in a single isolate was also found in quite a few numbers of previous molecular studies. In this analysis, 40.9 % of cefoxitin-resistant isolates exhibited multiple AmpC genes, of which blaFOX + ACC + CIT (22.2%) was the most common AmpC producer, while blaACC + CIT, blaACC + DHA, blaACC + MOX, blaFOX + CIT, blaFOX + DHA + CIT, blaFOX + DHA + CIT + MOX, blaFOX + ACC + CIT + MOX were also observed. A study from Tunisia identified multiple AmpC β-lactamases, including MOX + FOX + CMY-2-type enzymes, MOX + FOX-type enzymes and MOX + CMY-2-type enzymes, in *E. coli* and *K. pneumoniae* (18). Collectively, these geographical variations of the AmpC genes are of special scientific interest in the evolution of AmpC subtypes worldwide. Moreover, in diagnostic assays, molecular methods should be used for the identification, characterization, and epidemiological details of AmpC β-lactamases. In addition, these results also suggest that AmpC producers might be more widespread and can spread faster but cannot be diagnosed due to issues in the AmpC confirmation assays, close monitoring of these enzymes needs to be emphasized.

## CONCLUSION

The emergence of AmpC isolates is worrisome and underlines the necessity for further surveillance in this region. Furthermore, rational antimicrobial treatment

against these multidrug-resistant isolates and constant monitoring of antimicrobial resistance mechanisms among these isolates are recommended for optimal patient care. It is proposed that hospitals improve the surveillance of AmpC β-lactamase in clinical isolates and suggest using carbapenems to treat infections caused by AmpC-producing bacteria. In addition, carbapenem resistance should also be monitored strictly.

The limitations in our research were that we did not use molecular techniques to identify unique AmpC family genes in our isolates. The other drawback is that we have not been searching for other potential resistance mechanisms such as ESBL, MBL, efflux pump, etc.

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## CONFLICT OF INTEREST

Authors declare no conflicts of interest.

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