Original Article

# Detection of Plasmid-mediated Ampc β-lactamases Among E.coli and *Klebsiella pneumoniae by Multiplex PCR*

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## ABSTRACT

**Background:** Gram negative bacteria are acquiring drug resistance due to Extended spectrum beta lactamase (ESBL) production and also Plasmid mediated AmpC beta-lactamases (PMABLs). This is one of the major causes of multi-drug resistance among E.coli and Klebsiellainclinical practice. Detection of PMABL genes by molecular methods such as multiplex PCR gives accurate results in specific identification.

**Methods:** ESBL producing strains of 40 E.coli and Klebsiella were tested phenotypically for Plasmid mediated AmpC beta-lactamase production by using cefoxitin disk. The genes coding for PMABLs production was tested by multiplex PCR. Antibiotic susceptibility pattern of the isolates was also tested.

**Results:** 22(55%) of E.coli and 17(42.5%) of Klebsiella pneumoniae were phenotypically producing AmpC beta-lactamases. On genotypic testing 15(37.5%) E.coli and 11(28%) Klebsiella pneumoniae were positive for plasmid mediated AmpC beta-lactamases. Plasmid encoded AmpC genes in E.coli are CIT/EBC, CIT, and EBC. In Klebsiella pneumoniae the genes were CIT/DHA, CIT, and DHA. All the isolates showed 100% resistance to Cefoxitin and amox/clav and also higher degrees of resistance to cefotaxime, ceftazidime, cefepime, aztreonam and piperacillin/ tazobactam.

**Conclusion:** ESBL producing strains of E.coli and Klebsiella are developing drug resistance due to the production of PMABLs. Detection of genes coding for PMABL production are best tested by multiplex PCR which gives accurate results than phenotypic detection methods.

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## Introduction

The plasmid mediated AmpC β-lactamases (PMABLs) originate from the chromosomally located AmpC genes of several Gram negative bacteria. The productions of PMABL confers resistance to many β-lactam antibiotics including cephalosporins like cefotaxime, cefotetan, oxyimino-cephalosporins like cefotaxime, ceftazidime, ceftriaxone, also to monobactams like aztreonam and are not inhibited by clavulanic acid. [1, 2, and 3]

Horizontal gene transfer is the mechanism which is mainly found for the spread of antibiotic resistance genes. [4] The resistance exhibited by these plasmid mediated β-lactamase enzymes is rare, difficult to detect and they also have broad spectrum of resistance. They are of special concern because self-transmissibility permits their spread among different bacteria. [5, 6]

AmpC genes originate from *Hafniaalvei, Morganellamorganii, Citrobacterfreundii, Enterobacter cloacae* and two unknown organisms. [7]

The transferable AmpC gene products are commonly called plasmid mediated AmpC β-lactamases. [8, 9, and 10] PMABLs can be divided into five structurally distinct clusters: the *Citrobacterfreundii* cluster represented by CMY-2, the *Enterobacter spp*. Cluster with MIR-1 and ACT-1, the *Morganellamorganii* group with DHA-1, the *Hafniaalvei* cluster represented by ACC-1, the *Aeromonas spp*. Cluster with MOX-1 (also called CMY-1) and FOX-1 enzymes that constitute two distinct sub groups. [11]

Plasmid mediated AmpC β-lactamases cannot be reliably detected by standard susceptibility testing methods in the clinical laboratory.[12] Phenotypic tests are not reliable and may result in misreporting and treatment failures. The co-existence of ESBLs may also mask the phenotypic detection. Moreover there are no CLSI guidelines available for proper detection and confirmation of PMABL. (Black et al) [13] described the EDTA disk test and Young et al [14] described the "modified" Hodge test for detecting the presence of AmpC β-lactamases that could be carried out routinely in a busy clinical laboratory. Another test using boronic acid was described by Coudron. [15] However none of these tests can distinguish plasmid mediated hyper production of AmpC from chromosomal or any other mechanism of over production of an AmpC β-lactamase. Hence genotypic characterization is considered the gold standard. [11]

The present study was conducted to detect PMABLs in the clinical isolates of *Klebsiella pneumoniae* and *E.coli*, to characterize the genes encoding the pAmpC enzymes and also to determine their antibiotic susceptibility pattern.

## Materials and Methods

The study group comprised of40 ESBL producing isolates of *Klebsiella pneumoniae* and *E.coli*. The isolates were selected randomly and study was conducted for a period of 6 months. Ethical committee clearance was obtained. Organisms showing synergy between the amoxy/clav (30/10 mcg) disk and cefoxitin (30mcg) were considered as ESBL producing strains. Amp C production was phenotypically tested by using cefoxitin (30mcg) disc and zone diameter of <14mm was considered as resistant. Antibiotic susceptibility was determined by Kirby Bauer disk diffusion method for the following antibiotics: cefoxitin (30mcg), ceftazidime (30mcg), aztreonam (30mcg), amoxy/clav (30/10mcg), cefipime (30mcg), piperacillin/tazobatam (100/10 mcg), cefotaxime (30 mcg) (Supplied by Hi media laboratories, Mumbai, India.) *E.coli* ATCC 25922 strain was used as control and the results interpreted as per CLSI guidelines.

**Detection of Plasmid encoded AmpC genes:** PCR based genotyping assay was done to characterize the plasmid mediated AmpC β-lactamases using the primer sequences according to Hanson et al. [16]

The order of the work:

1. DNA sample preparation
2. Multiplex PCR
3. Genotype confirmation using PCR with individual primer sets

**DNA Sample Preparation:** The agar slant cultures were used to inoculate 3ml Luria-Bertani (LB) medium by scratching the surface of agar slant with a sterile micropipette tip. The tubes were kept in a rotator shaker at 37C 180rpm overnight. Next morning, an aliquot of 1ml was taken into micro centrifuge tube and centrifuged at 10,000rpm for 10min at room temperature. The supernatant was discarded and the pellet washed with water and finally suspended in 500ul deionized water. The suspension was boiled at $95^0$C for 20min to lyse the cells. It was then centrifuged and the supernatant was used as a source of bacterial DNA for the PCR.

**Multiplex PCR:** The PCR reactions were initially carried out in a Multiplex pattern with the six pairs of primers. The primers used for PCR amplification are listed below.

PCR was done as follows:
Total reaction volume: 5ul
Template: 0.5ul crude lysate
10X PCR buffer (containing 17.5mM MgCl2): 0.5ul
dNTPs: 0.25ul (containing 2.5mM each)
Primer: 0.25ul each from a 10pM stock
Taq Polymerase (From Himedia): 0.5U

**Cycling Conditions:**

1. Initial setting at95°C for 5min
2. Second step includes 35 cycles of 95°C for 15sec, 62°C for 15 sec, 72°C for 45sec
3. Followed by a final extension at 72°C for 5min.

The products were analysed on 2% agaroseTris-acetate-EDTA gels pre - stained with ethidium bromide.

The samples which showed a band in multiplex PCR were further analysed using the individual primer sets to confirm the result.

In addition to the regular end-point PCR, a quick PCR method was also checked (According to Hansen et al). The composition of the PCR mixture for the rapid PCR was the same except the buffer contains magnesium sulphate (at 4mM concentration) instead of Magnesium chloride.

**The cycling conditions for the rapid PCR are as follows**.

1. Initial temperature 95°C for 30sec
2. Second step includes 35 cycles of 95°C for 5 sec, 58°C for 10sec, 72°C for 10sec
3. Followed by a final extension at 72°C for 10sec

The products were analysed on 2% agaroseTris-acetate-EDTA gels prestained with ethidium bromide.

**Normal (singlet) PCR:** The samples which showed a band in multiplex PCR were further analysed using the individual primer sets to confirm the result. In addition a quick PCR method was also checked (according to Hanson et al)..

The sequences of the primers are as follows: (supplied by Bio serve pvt ltd, Hyderabad, India.)

| | | |
|---|---|---|
| MOXMF | 5′ GCT GCT CAA GGA GCA CAG GAT 3′ | (21 bases) |
| MOXMR | 5′ CAC ATT GAC ATA GGT GTG GTG C 3′ | (22 bases) |
| CITMF | 5′ TGG CCA GAA CTG ACA GGC AAA 3′ | (21 bases) |
| CITMR | 5′ TTT CTC CTG AAC GTG GCT GGC 3′ | (21 bases) |
| DHAMF | 5′ AAC TTT CAC AGG TGT GCT GGG T 3′ | (22 bases) |
| DHAMR | 5′ CCG TAC GCA TAC TGG CTT TGC 3′ | (21 bases) |
| ACCMF | 5′ AAC AGC CTC AGC AGC CGG TTA 3′ | (21 bases) |
| ACCMR | 5′ TC GCC GCA TC ATC CCT AGC 3′ | (21 bases) |
| EBCMF | 5′ TCG GTA AAG CCG ATG TTG CGG 3′ | (21 bases) |
| EBCMR | 5′ CTT CCA CTG CGG CTG CAA GTT 3′ | (21 bases) |
| FOXMR | 5′ AAC ATG GGG TAT CAG GGA GAT G 3′ | (22 bases) |
| FOXMR | 5′ CAA AGC GCG TAA CCG GAT TGG 3′ | (21 bases) |

**Statistical methods:** All data was kept in terms of number of cases and percentages. P value < 0.05 was considered statistically significant. Microsoft Excel 2007 and SPSS were used to do the calculations.

## Results

40 ESBL producing strains of *E.coli* and *Klebsiella pneumoniae* were tested for cefoxitin resistance and were considered as putative AmpC producers. Out of40, 22(55%) *E.coli* and 15(37.5%) *Klebsiella pneumoniae* were phenotypically positive for Amp C production. Table no.1

Plasmid mediated AmpC genes were detected by Multiplex PCR in the phenotypically positive *E. coli* and *Klebsiella pneumonia* strains. The plasmid mediated AmpC genes detected in *E.coli* belonged to CIT and EBC families. In *Klebsiella pneumonia* strains the genes belonging to CIT and DHA were detected. No genes belonging to FOX, MOX, and ACC were detected. Table no. 2

Figure 1, 2, 3 and 4 shows the gel pictures of the Multiplex and individual PCR products.

Antibiotic susceptibility testing showed that all PMABL producing strains were resistant to aztreonam (80%), amoxy/clav (100%), cefotaxime (85%), piperacillin/tazobatam (50%), cefipime (100%), cefoxitin (100%), and ceftazidime (50%). Table no.3

**Table 1: Total no. of isolates showing phenotypic and genotypic result for PMABLs**

| organism | Phenotypic positive | Genotype positive |
|---|---|---|
| E.coli (n= 40 ) | 22(55%) | 15 (37.5%) |
| Klebsiella pneumonia (n= 40 ) | 17 (42.5%) | 11(28%) |

**Table 2: Types of AmpC genes in each organism**

| Organism (total no. of positive isolates) | Plasmid encoded AmpC gene types |
|---|---|
| E.coli ( 15) | CIT/EBC , CIT , EBC |
| Klebsiella pneumoniae ( 11 ) | CIT/DHA , CIT , DHA |

**Table 3: Antibiotic resistance pattern of E.coli and Klebsiella pneumoniae with reference to AmpC production CX- cefoxitin, CTX-cefotaxime, CAZ- Ceftazidime, FEP-cefepime, AT-aztreonam, AMC- Amoxy/clav, p/taz-piperacillin/tazobactam (Disks obtained from Hi media, Mumbai, India)**

| Antibiotic / Organism | CX 30µg | CTX 30µg | CAZ 30µg | FEP 30µg | AT 30µg | AMC 30/10µg | PIT 100/10µg |
|---|---|---|---|---|---|---|---|
| E.coli (n= ) | 100% | 85% | 78% | 72% | 80% | 100% | 67% |
| Klebsiella pneumoniae | 100% | 79% | 63% | 68% | 86% | 100% | 50% |

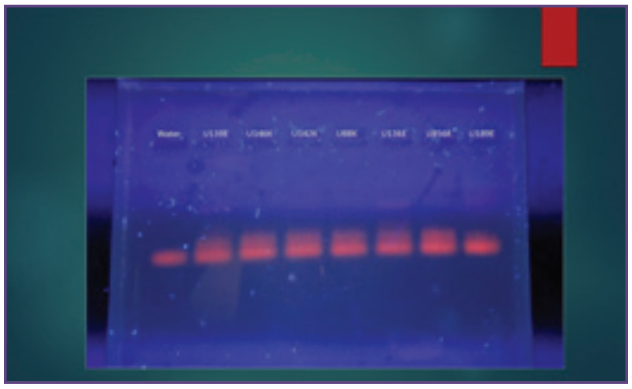

**Fig. 1: Gel Picture Showing PCR Amplification of CIT, EBC and DHA Genes**

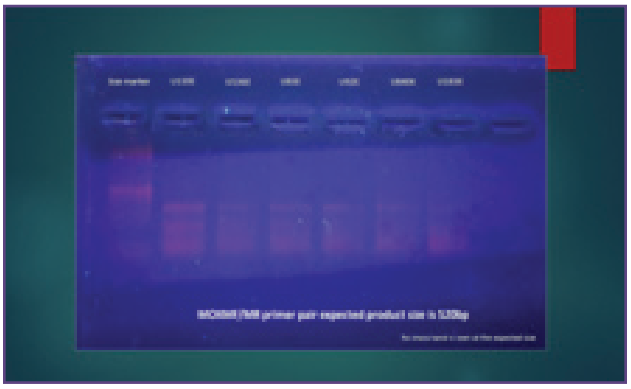

**Fig. 2: Gel Picture Showing PCR Amplification of MOX Genes**

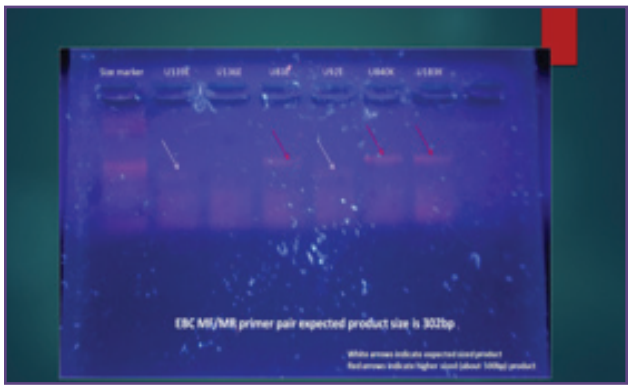


**Fig. 3: Gel Picture Showing PCR Amplification of EBC Genes**

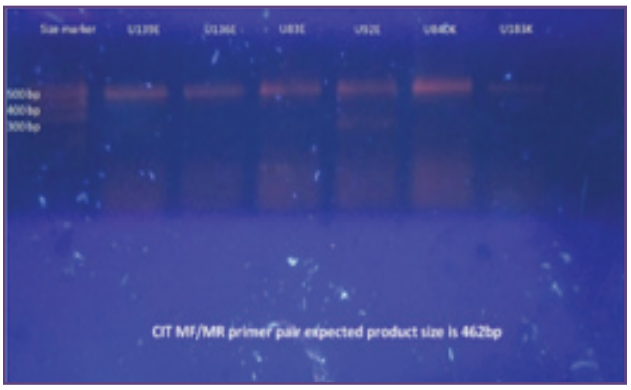


**Fig. 4: Gel Picture Showing PCR Amplification of CIT Genes**

## Discussion

Beta lactamases continue to be the leading cause of resistance to β-lactam antibiotics in gram negative bacteria. There has been an increased incidence and prevalence of ESBLs, the enzymes that hydrolyse and cause resistance to oxyimino-cephalosporins and aztreonam. [17] Compared to ESBL producers, isolates producing AmpC β-lactamase are resistant to additional β-lactams and insusceptible to currently available β-lactam inhibitors and have the potential for developing resistance to carbapenems. [18]

In India AmpC producing strains of Enterobacteriaceae have emerged as a challenge in hospitalised as well as community based patients. [19]

In our study 55% of *E.coli* and 42.5% of *Klebsiella pneumoniae* strains were detected to be AmpC β-lactamase producers phenotypically .Various studies from different parts of the country during the last decade AmpC production has been reported . From Delhi 6.9% of *E.coli* and 6.18% of *Klebsiella pneumonia*, [20] from some parts of Kolkata 47.8% of *E.coli* and 13% of *Klebsiella spp*. were reported as AmpC β-lactamase producers. [21] 3.3% of *E.coli* and 2.2% of *Klebsiella spp*. from Karnataka and 3.4% of *E.coli* and 4.8% of *Klebsiella spp*. from some regions of Andhra Pradesh were found to have AmpC enzymes. [22, 23, 24, and 25] This large difference and variation may be due to the difference in the selection criteria of isolates, the

variation in the ability to produce AmpC β-lactamases among different Gram negative bacteria, different clinical specimens and also it's prevalence in different geographical areas. More over the studies based on phenotypic detection cannot differentiate between the plasmid mediated enzyme producers and chromosomal hyper producers or porin loss mutants. The phenotypic studies could not differentiate the types or families of plasmid mediated AmpC β-lactamase. [7, 26] This recent increase in AmpC producing isolates indicates that more and more isolates are acquiring resistant mechanisms making the antibiotic treatment ineffective. [27]

Hence there is a need to use molecular identification methods to detect and distinguish AmpC- mediated resistance from other β-lactamase resistance mechanisms. Differentiation between these types of organisms would prevent the unnecessary usage of cephalosporins and carbapenems which ensures effective therapeutic intervention and optimal clinical outcome. [28, 29]

In our study multiplex PCR was used for the detection of family specific AmpC genes ACC, FOX, MOX, DHA, CIT andEBC in *E.coli* and *Klebsiella pneumoniae*. Out of 22 (55%) of phenotypically AmpC positive strains of E.coli 15 (37.5%) showed genotypes CIT and CIT/EBC. out of 17(42%) of phenotypically AmpC positive strains of *Klebsiella* 11(28%) showed the Geno types CIT, CIT/DHA. In one study by HaengSJ *et* al, 22 *Klebsiella pneumonia* isolates showed DHA type. [30]

In one study by Neil Woodford *et al* CIT genotype was found in *E.coli* strains and DHA genotype was found in *Klebsiella spp*. [31] correlating with our study.

Shahidet al has demonstrated the occurrence of CIT, EBC and CIT/EBC ($bla_{CIT}$and$bla_{EBC}$) in both E.coli and Klebsiella spp. by using multiplex PCR [32] which is correlating with our study.

In one study by Shanti et al detected CIT, EBC and DHA family specific genes along with other types. [33]

In our study AmpC producing *E.coli* and *Klebsiella* isolates showed 100% resistance to cefoxitin and amoxy/clav which may be due to hyper production of β-lactamases and inhibitor resistant TEM β-lactamases. Resistance to cefoxitin can also indicate the reduced outer membrane permeability. [34]

All AmpC producers showed high resistance to aztreonam (80%), cefotaxime 79%, ceftazidime 63%, cefipime (68%) and piperacillin/tazobatam (67 %.).In a study by Sasirekhaet al there was high resistance to amoxy/Clav, aztreonam 42.85% of resistance to cefepime. [27]

In a study by Renukaet al showed high resistance pattern 81.63% to amoxy/Clav, cefpodoxime 72.44%, aztreonam 67.34%, and piperacillin / tazobatam (69%) and were multidrug resistant .[35]

In view of the above findings there is a need to detect drug resistant strains to prevent the spread of drug resistance in hospitals as well as in the community.

Limitations of our study: In the present study PMABL genes were detected but other mechanisms of cefoxitin resistance such as porin loss mutants and chromosomal hyper producers were not considered and detected.

## Conclusions

The most important aspect for a clinical microbiologist is detection of PMABLs and their susceptibility pattern among gram negative organisms. In our study ESBL producing *Klebsiella* and E.coli strains showed PMABL CIT, EBC, CIT/EBC, DHA, CIT/DHA genes by multiplex PCR. These strains with AmpC genes are often resistant to multiple antimicrobial agents making it difficult to select an effective antibiotic. To detect AmpC resistance clinical laboratories will need to use combination of phenotypic and molecular identification methods. The multiplex PCR technique described in this study will be an important tool for the detection of PMABL genes in Gram negative bacteria.

## Abbreviations

**PMABL:** Plasmid mediated AmpC beta- lactamase

**ESBL:** Extended spectrum beta- lactamase

**PCR:** Polymerase chain reaction

**EDTA:** Ethylene diamine tetra acetic acid

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## Competing Interests

None Declared

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