Indian J Med Res 135, January 2012, pp 114-119

Molecular description of plasmid-mediated AmpC β-lactamases among nosocomial isolates of *Escherichia coli* & *Klebsiella pneumoniae* from six different hospitals in India

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Received May 4, 2010

***Background & objectives*: Plasmid mediated AmpC β-lactamase (PMABL) resistance in *Escherichia coli* and *Klebsiella* spp. is an emerging problem worldwide. Phenotypic methods are commonly used for detection of PMABL production in Gram-negative isolates, but molecular data about the prevalence of plasmid-mediated AmpC-type resistance at the national level are needed. Hence, a prospective study was undertaken to determine the occurrence of PMABL gene and its types among clinical isolates of *E. coli* and *K. pneumoniae* obtained from six different hospitals in India.**

***Methods*: A total of 241 nosocomial isolates of *K. pneumoniae* (n=109) and *E.coli* (n=132) from six geographically distant hospitals in India were included. These were screened for cefoxitin resistance. AmpC disk test and modified three dimensional extraction test were used for phenotypic detection of PMABL production. Molecular types were determined by a multiplex PCR.**

***Results*: Among the 241 isolates, 187 (77.5%) were found to be cefoxitin resistant (*K. pneumoniae* n=83, *E. coli* n=104). AmpC activity was detectable in 153 (63.4%) isolates, (*K. pneumoniae* n=69, *E. coli* n=84). By PCR, the plasmid encoded AmpC genes were found in 92 (38.1%) isolates and the molecular types of the genes detected predominantly were DHA, CIT followed by MOX and ACC types.**

***Interpretation & conclusions*: A high percentage of plasmid-encoded AmpC enzymes was noted in *E. coli* and *K. pneumonia* isolates obtained from different parts of the country. Phenotypic methods alone may not reflect the true number of PMABL producers. Genotypic methods need to be employed in national surveillance studies.**

Key words *Escherichia coli* - Indian isolates - *Klebsiella pneumoniae* multidrug resistance - multiplex PCR - plasmid-mediated AmpC β- lactamases

AmpC β-lactamase production is one of the mechanisms of resistance to β-lactam antibiotics in Gram negative bacteria conferring resistance to a wide variety of β-lactam antibiotics including 7-α-methoxy cephalosporins (cefoxitin or cefotetan), oxyimino cephalosporins (cefotaxime, ceftazidime, ceftriaxone), monobactam (aztreonam) and are not inhibited by clavulanic acid[1]. These are of two types, chromosomal inducible and plasmid mediated non-inducible. Plasmid mediated AmpC β-lactamases (PMABLs)

have evolved by the movement of chromosomal genes on to plasmids and are found in *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella* spp, *Proteus mirabilis*, *Citrobacter freundii*, *Enterobacter aerogenes* which confer resistance similar to their chromosomal counterparts. Currently, there are over 30 known types derived from *Enterobacter cloacae*, *Morganella morganii*, *Citrobacter freundii, Hafnia alvei* and other of unknown origin[2].

Organisms producing PMABLs such as *E.coli* and *Klebsiella* spp, are often associated with multidrug resistance, leaving a few therapeutic options. PMABLs can be detected by various phenotypic methods such as Amp C disk test[3], three dimensional test[4], cefoxitin agar method[5], modified double disk test[6], using inhibitors like boronic acids[7], syn2160 compunds[8] and broth micro dilution method[7]. Testing PMABL is not widely attempted by many laboratories; phenotypic tests may be ambiguous and unreliable resulting in misreporting and treatment failures. In addition, the co-existence of extended spectrum β-lactamases (ESBLs) may mask its detection phenotypically. There are no Clinical Laboratory Standards Institute (CLSI) guidelines available for its optimal detection and confirmation[9]. Phenotypic tests do not differentiate between chromosomal AmpC genes and AmpC genes that are carried on plasmids. Hence, genotypic characterization is considered as the gold standard[10].

In view of the increasing reports of PMABL producing strains of *Klebsiella* spp. and *E. coli* and its types from around the world, and paucity of molecular studies in our country, the present work was conducted to examine the occurrence of PMABL gene types among the nosocomial isolates of *K. pneumoniae* and *E.coli* from six different hospitals.

Material & Methods

Bacterial isolates: In this study, during a period of five months (September 2009-January 2010), a total of 241 non-duplicate clinical isolates of *K. pneumoniae* (n=109) and *E.coli* (n=132) from hospitalized patients obtained from six geographically distant hospitals in India were investigated (Table I). All the isolates were identified as per the standard bacteriological procedures[11], stocked in 0.2 per cent semi-solid agar tubes and transferred to the Microbiology Department of the study centre Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry for further characterization. This study was restricted to *E. coli* and *K. pneumoniae* isolates only as the CLSI guidelines for ESBL screening and confirmation are available only for these two[9].

Antibiotic susceptibility testing: Antibiotic susceptibilities were determined by the standard disk diffusion test for the following antibiotics (concentration/disk in µg); ceftazidime (30), ceftriaxone (30), cefotaxime (30), cefoxitin (30), aztreonam (30), amikacin (30), tetracycline (30), ampicillin (10), co-trimoxazole (10), cefepime (30), gentamicin (10), meropenem (10), imipenem (10), ciprofloxacin (5), amoxycillin-clavulanic acid (30/10), piperacillin-tazobactam (100/10) (Hi-Media, Mumbai, India). *E. coli* ATCC 25922 was used as the control and the results were interpreted as per CLSI criteria[9].

Isolates showing resistance to cefoxitin (inhibition zone <18 mm), a 3rd generation cephalosporins (3GC), intermediate or resistant to amoxycillin-clavulanic acid and showing no cephalosporin/clavulanate synergism were considered as putative AmpC producers.

Amp C disk test: AmpC disk test was done for the phenotypic detection of AmpC β-lactamases production on a Muller-Hinton agar (MHA) plate as described previously[3]. A flattening or indentation of the cefoxitin inhibition zone in the vicinity of the disk with test strain was interpreted as positive for the production of AmpC β-lactamase. An undistorted zone was considered as negative.

Table I. Distribution and source of the clinical isolates from various parts of the country

S. no	Collection hospital, State	Total no. of isolates	*K. pneumoniae*	*E. coli*
1	JIPMER Hospital, Puducherry	81	47	34
2	PSG Medical College Hospital, Coimbatore, Tamil Nadu	20	-	20
3	Apollo Hospitals, Mysore, Karnataka	37	7	30
4	Pandit BT Sharma Postgraduate Institute of Medical Sciences, Rohtak, Haryana	40	20	20
5	Government Medical College Hospital, Chandigarh	18	14	4
6	Indira Gandhi Medical College, Shimla, Himachal Pradesh	45	21	24

Modified three-dimensional test: Plasmid mediated AmpC beta lactamases production was further confirmed by the modified three-dimensional test[12]. Briefly, crude enzyme extract of the test organism was prepared by repeated freeze thawing in -80°C for seven times. A lawn culture of *E. coli* ATCC 25922 was made on MHA and a cefoxitin (30 µg) disk was placed at the centre. Linear slit was cut, 3 mm away from the disk and 30 µl of the enzyme extract was added to a well made at the outer edge of the slit. The plate was incubated overnight at 37°C. Clear distortion of zone of inhibition of cefoxitin is considered as positive test. Quality control was achieved by using known in-house AmpC positive isolate of *K. pneumoniae*.

Detection of Plasmid encoded AmpC genes: All the isolates that were phenotypically positive for AmpC activity were tested by a multiplex PCR assay which identifies six family-specific AmpC genes carried on the plasmids such as MOX, FOX, EBC, ACC, DHA and CIT[13]. Template DNA was obtained by boiling lysis method. Amplification was performed on a Corbett Research thermal cycler (HP, USA) as per primers and condition described[13]. ACC, DHA and CIT positive *E.coli* controls were included (Kindly provided by Dr. John Hays, Erasmus Medical College, The Netherlands) and the PCR products were analyzed by electrophoresis in 2 per cent agarose gels stained with ethidium bromide (Fig. a & b).

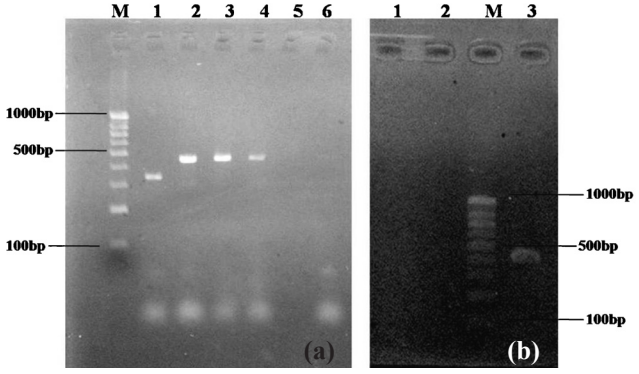

Fig. PCR for Plasmid-encoded AmpC β-lactamase genes: 2 per cent Agarose gel showing products of PCR amplification. **(a)** M-100bp molecular size standard DNA ladder. Lane 1- *E.coli* with 342bp ACC gene, Lane 2- *K. pneumoniae* with 462bp CIT, Lane 3 & 4 -*E. coli* showing 462bp CIT gene, Lane 5- negative *K. pneumoniae* & Lane 6- negative water control. **(b)** Lane 1- negative *K. pneumoniae*, Lane 2- negative water control, M-100bp molecular size standard DNA ladder, Lane 4- *E.coli* with 405 DHA positive, the 520bp *MOX* gene was not shown in the figure.

Results

Of the 241 isolates tested, 187 (77.5%) were cefoxitin resistant (*K. pneumoniae* n=83, *E. coli* n=104) and were thus considered as putative AmpC producers. Phenotypically, AmpC production was present in 63.4 per cent (153/241) isolates (*K. pneumoniae* n=69, *E. coli* n=84). Using AmpC disk test and modified three-dimensional tests, PMABL production was detected in 137 (73.2%) and 149 (79.6%) of cefoxitin resistant isolates, respectively.

Among the 241 total isolates tested, plasmid-encoded AmpC genes were detected by PCR in 92 (38.1%), which included *K. pneumoniae* (n=32) and *E. coli* (n=60). Of these, plasmid-encoded AmpC genes belonging to the DHA family were detected in 43 (46.7%) isolates. Of the 43 DHA positive isolates, 19 (44.1%) were *K. pneumoniae* and 24 (55.8%) were *E. coli*. Plasmid-encoded AmpC genes belonging to the CIT family were detected in 38 per cetn (35/92) isolates[24]. *E. coli* isolates (68.5%) and 11(31.4%) *K. pneumoniae*.

AmpC genes belonging to the MOX family were detected in 14.1 per cent (13/92) isolates, [*E. coli* (n=11, 84.6%) and *K. pneumoniae* (n=2, 15.3%)]. Gene of the family ACC type was present in only one isolate. No genes belonging to the FOX or EBC family were detected.

Of the 92 isolates identified as possessing plasmid-mediated AmpC, the distribution of the sources were from blood cultures (n=17, 18.4%), urine (n=44, 47.8%), and other body fluids (n=31, 33.6%).

Among the 84 *E. coli* that showed AmpC production phenotypically, non-plasmid-derived AmpC activity was present in 28.5 per cent (n=24). Similarly, among the 69 *K. pneumoniae* isolates non-plasmid-derived AmpC activity was present in 53.6 per cent (n=37).

By antibiotic susceptibility testing, all the PMABL producers were resistant to piperacillin/tazobactam, amoxycillin/clavulanate combination and 84 (91%) were resistant to co-trimoxazole, gentamicin, tetracycline and amikacin thus showing multidrug resistance. Among the PMABL producers, (67%) had shown cefepime resistant. A total of 26 (10.7%) and 13(5.3%) isolates were resistant to meropenem and imipenem, respectively.

Discussion

Plasmid mediated AmpC β-lactamases represent a new threat since these 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 in *E.coli* and *K. pneumoniae* has been found around the world causing nosocomial outbreaks[14,15].

Distinguishing between cefoxitin-resistant AmpC producers from cefoxitin-resistant non-AmpC producers could guide treatment options, *i.e*. extended spectrum cephalosporins for cefoxitin-resistant non-AmpC, non-ESBL producers and carbapenems for the cefoxitin-resistant AmpC producers. Differentiation between these types of organisms would prevent the unnecessary usage of cephalosporins and carbapenems resulting in the selective pressure driving the AmpC or plasmid mediated class A carbapenem resistance gene propagation[16]. There are also concerns that treatment failures will occur with certain cephalosporins due to incorrect susceptibility tests when organisms producing PMABL appears falsely susceptible. Hence, detection of PMABL producing organisms is important to ensure effective therapeutic intervention and optimal clinical outcome[8,17].

In this study, occurrence of a large percentage of multidrug resistance has been observed among the PMABL producing strains. Though AmpC producers are susceptible to tazobactam compared to other β-lactamases inhibitors, a high resistant to piperacillin/tazobactam combination was noticed in our isolates, which may be due to hyperproduction of β-lactamases and inhibitor-resistant TEM β-lactamases, which are responsible for resistance to inhibitor combinations among *E. coli* and *K. pneumoniae*[1]. Further, the co-presence of ESBLs adds to the mechanism of resistance to piperacillin/tazobactam and cefepime (Data not shown). The resistance to piperacillin/tazobactam among the AmpC producers in our study was similar to the findings of a study from south India[18] and contrary to that of Taneja *et al*[19]. With the continuing use of cefoxitin and cefotetan and the clinical introduction of β-lactamase combinations such as clavulanate with amoxicillin or ticarcillin, sulbactam with ampicillin, and tazobactam with piperacillin, plasmids encoding class C β-lactamases appeared. Such enzymes provide a broader spectrum of resistance than ESBLs[1] .

In the last one decade, AmpC production has been reported from various parts of the country. From north India, 6.97 per cent *E.coli* and 6.18 per cent *K. pneumoniae* (New Delhi)[20], 9.9 per cent *E. coli* and 31.1 per cent *K. pneumoniae*[21] were reported as PMABL producers. From eastern part of the country (Kolkata) 47.8 per cent *E. coli*, 13 per cent *K. pneumoniae* were reported as AmpC β-lactamase producers[22]. From southern States, 24.1 per cent of *Klebsiella* spp. and 37.5 per cent of *E. coli* (Chennai), 9.2 per cent was reported in an another study from Chennai; 3.3 per cent of *E. coli*, 2.2 per cent of *K. pneumoniae* (Karnataka) and 3.4 per cent of *E. coli*, 4.8 per cent of *K. pneumoniae* (Andhra Pradesh) were found to harbour AmpC enzymes[19,23-25]. However, these studies were based on phenotypic tests which do not differentiate between the plasmid-mediated enzymes producers and the chromosomal hyper producers or porin loss mutants. Also these studies did not differentiate the types or families of plasmid-mediated AmpC β-lactamase. Thus, molecular studies will help us to know the actual prevalence of these enzymes[13, 27].

It is pertinent to note that in this study 10.4 per cent of the cefoxitin resistant isolates were not detected by AmpC disk test and 2.6 per cent by modified three dimensional test. Phenotypic tests alone may not reflect the true number of PMABL producers, hence, molecular studies, although not possible routinely in clinical laboratories, need to be employed in surveillance studies.

The knowledge about the molecular types and the prevalence of plasmid-mediated AmpC-type resistance at the national level is important to provide useful information needed for targeted antimicrobial therapy and better infection control[16]. In this study, of the 241 isolates tested, 92 carried plasmid-encoded AmpC genes (38.1%), with an occurrence of 29.3 per cent in *K. pneumoniae* and 45.5 per cent in *E.coli* thus showing their presence among Indian isolates.

In a nationwide study from China[26], the prevalence of plasmid-mediated AmpC β-lactamases was 10.1 per cent in *K. pneumoniae* and 2.0 per cent in *E. coli* strains. Similarly, from the United States[27], 8.5 per cent of the *Klebsiella* spp. and 4 per cent of the *E. coli* strains contained plasmid-mediated AmpC-type enzymes. From Singapore[28], 26 per cent of PMABL genes were reported and the lowest rates of AmpC genes were reported from Switzerland as 0.2 per cent in *E. coli* and 0.4 per cent in *K. pneumoniae*[29]. Thus, the percentage of PMABL genes production was found to be higher in our country compared to China, US and Singapore and Switzerland. On the contrary, compared to our results, the highest prevalence of AmpC genes were reported in a Korean surveillance[30] showing 73 per cent of *E. coli* and 77 per cent of *K. pneumoniae* carrying PMABL genes.

Table II. Distribution of the types of plasmid-encoded AmpC β-lactamase genes from various region of the country

Region	Organism (total no. of isolates)	Plasmid- encoded AmpC genes types n(%)
Puducherry	*K. pneumoniae* (47)	CIT 6 (12.7), DHA 5 (10.6), MOX 1 (2.1)
	E. coli (34)	CIT 9 (26.4), DHA 3 (8.8), MOX 3 (8.8)
Coimbatore	*K. pneumoniae* (0)	ND
	E. coli (20)	CIT 9 (45), DHA 3 (15)
Mysore	*K. pneumoniae* (7)	NP
	E. coli (30)	CIT 2 (6.6),DHA 5 (16.6), MOX 4 (13.3), ACC 1(3.3)
Haryana	*K. pneumoniae* (20)	CIT 1 (5), DHA 6 (30)
	E. coli (20)	CIT 1 (5), DHA 7 (35), MOX 2 (10)
Chandigarh	*K. pneumoniae* (14)	CIT 1 (7.1), DHA 4 (28.5), MOX 1 (7.1)
	E. coli (4)	DHA 1 (25), MOX 1 (25)
Shimla	*K. pneumoniae* (21)	CIT 3 (14.2), DHA 4 (19)
	E. coli (24)	CIT 3 (12.5), DHA 5 (20.8), MOX 1 (4.1)
Total	*K. pneumoniae* (109)	CIT 11 (10), DHA 19(17.4), MOX 2(1.8)
	E. coli (132)	CIT 24 (18.1), DHA24 (18.1), MOX 11(8.3), ACC 1 (0.7)

(n)= n(%)= total number of positive isolates (percentage); CIT,DHA,MOX, ACC indicate the plasmid encoded AmpC gene types. NP- none positive, ND- test not done

Plasmid mediated AmpC β-lactamases from *K. pneumoniae* isolates was first reported in 1989 in Seoul, South Korea[14]. Subsequently, these have been reported worldwide and 29 different plasmid-mediated AmpC genes have been identified to date and deposited in Gene Bank[15]. In 1998, CMY-4 was reported from India from a strain of *K. pneumoniae*[1] and CMY-6 type (CIT family) was reported in 2009 from Uttar Pradesh[21]. In the present study, DHA and CIT type genes were predominantly present in *K. pneumoniae* and *E.coli*, followed by MOX and ACC types in *E.coli* (Table II). The ACC genes were recovered only from the southern part of the country. Whereas, FOX or EBC family genes were not detected from any region. The current study was restricted mainly to detect plasmid mediated AmpC gene types and hence the co-existence of ESBLs was not shown.

This study had certain limitations. The presence of only plasmid mediated AmpC β-lactamase genes was targeted and the other possible mechanisms of cefoxitin resistance like chromosomal hyper producers or porin loss mutants were not detected. Further, we have come across strains with imipenem and cefoxitin resistance and the possible mechanism of this phenotype could be due to the presence of carbapenemases like *K. pneumoniae* carbapenemase (KPCs) along with AmpC[31] and their occurrence were not looked for.

In conclusion 38.1 per cent isolates of *K. pneumoniae* and *E. coli* showed the occurrence of PMABL which is alarming. Dissemination of these organisms within the hospital or between the different regions of the country may become an important public health issue. Hence, identification of types of AmpC may aid in hospital infection control and help the physician to prescribe the most appropriate antibiotic, thus decreasing the selective pressure, which generates antibiotic resistance. Further studies such as sequencing and typing the strains may be required to better understand the genetic relatedness and the molecular epidemiology of this resistance mechanism.

Acknowledgment

The authors acknowledge the following contributors from various parts of the country who provided the clinical isolates for this study. Dr Jagdish Chander, Government Medical College Hospital, Chandigarh; Dr Puneeth Kumar Gupta, Indira Gandhi Medical College, Shimla; Dr Uma Chaudhary, Pandit BD Sharma PGIMS, Haryana; Dr Chaya, Apollo Hospitals, Mysore and Dr Appalaraju, PSG Medical College, Coimbatore. Part of the work was funded by University Grants Commission, New Delhi.

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