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Phenotypic & molecular characterization of AmpC β-lactamases among *Escherichia coli, Klebsiella* spp. & *Enterobacter* spp. from five Indian Medical Centers

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***Background & objectives*: AmpC β-lactamases which are often plasmid mediated hydrolyze all β-lactam antibiotics except cefepime and carbapenems. We evaluated the presence of AmpC β-lactamases among *Enterobacteriaceae* strains recovered prospectively from patients at five Indian tertiary care centres.**

***Methods*: The study included 909 consecutive Gram-negative isolates recovered from clinically significant specimens during June 2007 - May 2008 as part of an ICMR-ESBL study. Among the study isolates, 312 were found to be cefoxitin resistant by disc diffusion test (DDT). Minimum inhibitory concentration (MIC) determination by E test was done against amikacin, levofloxacin, impinem, meropenem, ertapenem, tigecycline and piperacillin-tazobactam. Combined DDT using phenyl boronic acid as inhibitor with cefoxitin was used for phenotypic confirmation of AmpC phenotype. The common Amp C genotypes *ACC, FOX, MOX, DHA, CIT* and *EBC* were detected by multiplex PCR.**

***Results*: Plasmid mediated Amp C phenotype was confirmed in 114 of the 312 (36.5%) cefoxitin resistant isolates with 255 (81.7%) showing multidrug resistance. Susceptibility to tigecycline was highest (99%) followed by imipenem, meropenem (97%), ertapenem (89%), amikacin (85%), and piperacillin-tazobactam (74.6%). Levofloxacin resistance was 82 per cent. ESBL co carriage was observed among 92 per cent of Amp C producers. Among 114 Amp C producers, 48 could be assigned a genotype, this included CIT- FOX (n=25), EBC (n=10), FOX (n = 4), CIT (n=3), EBC-ACC (n=2) and one each of DHA, EBC-DHA, FOX –DHA and FOX-EBC-DHA.**

***Interpretation & conclusions*: Overall, AmpC phenotypes were found in 12.5 per cent isolates, multidrug resistance and ESBL co-carriage among them was high suggesting plasmid mediated spread. The study results have implications in rational antimicrobial therapy and continued surveillance of mechanisms of resistance among nosocomial pathogens.**

Key words AmpC β-lactamases - Amp C genotypes - cefoxitin resistance - ESBL - multidrug resistance

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AmpC β-lactamases are clinically important cephalosporinases produced by many *Enterobacteriaceae* strains and mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins and β-lactam/β-lactam inhibitor combinations[1]. Plasmid-mediated AmpC genes first reported in 1988, constitute an emerging therapeutic problem[2]. The plasmid-mediated AmpC genes are derived from inducible chromosomal genes that have become mobilized. Commonly reported genotypes are ACC, FOX, MOX, DHA, CIT and EBC[3-5]. These enzymes confer a resistance pattern similar to the overproduction of chromosomal AmpC β-lactamases, which may involve all β-lactam antibiotics except for carbapenems and cefepime[6].

In many bacteria, AmpC enzymes are inducible and can be expressed at high levels by mutation. Overexpression confers resistance to broad-spectrum cephalosporins including cefotaxime, ceftazidime, and ceftriaxone and is a problem especially in infections due to *Enterobacter aerogenes* and *E. cloacae*, where an isolate initially susceptible to these agents may become resistant upon therapy[7]. Transmissible plasmids have acquired genes for AmpC enzymes, which consequently can now appear in bacteria lacking or poorly expressing a chromosomal AmpC gene, such as *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis*. Resistance due to plasmid mediated AmpC enzymes is less common than ESBL production in most parts of the world but may be both harder to detect and broader in spectrum[7].

Plasmid-mediated AmpC genes are of special interest because their mobility allows them to emerge in one genus or species and spread to different organisms. The prevalence of plasmid mediated AmpC-type resistance at the national level in most countries is unknown because studies have not examined the strains at the molecular level required to elucidate the different mechanisms involved. A 2004 report from the United States documented 7 to 8.5 per cent of the *Klebsiella* spp. and 4 per cent of the *Escherichia coli* isolates contained plasmid mediated AmpC type enzymes[8]. In a 2005 Canadian study, 13.5 per cent of the isolates harboured a gene that correlated with acquired AmpC CMY-2 type resistance, and in all strains the gene was identified as CMY-2[9]. In 2006, a study from US reported the rate of transferable AmpC producer to be 3.3 per cent with *FOX-5* genes most predominantly occurring in *K. pneumoniae*[10]. Plasmid mediated AmpC was present in 26 per cent of study isolates, with CMY like enzymes detected predominantly in *E.coli* and DHA like enzymes predominantly in *K. pneumoniae* in a study from Singapore[11].

In India, AmpC prevalence has been reported in *Klebsiella* spp (24.1%) and *E coli* (37.5%)[12]. In another Indian study, 3.3 per cent of isolates produced AmpC β lactamases[13]. In this report, we present the detailed phenotypic and molecular characterization of prospectively collected cefoxitin-resistant *E. coli, Klebsiella* spp. and *Enterobacter* spp. from a multicentric Indian survey. The usefulness of a screening test using phenyl boronic acid as inhibitor of AmpC enzymes was evaluated.

Material & Methods

Study design: In this prospective laboratory based surveillance study (June 2007 to May 2008), 909 non repeat Gram-negative strains [*E.coli* (n=517), *Klebsiella* spp. (n=331) and *Enterobacter* spp. (n=61)] determined to be clinically significant [skin and soft tissue (132), blood (n= 91), and urinary tract infection (89)] were collected from five Indian tertiary care centres All India Institute of Medical Sciences (AIIMS), New Delhi, Amrita Institute of Medical Sciences (AIMS), Kochi, Sanjay Gandhi Post Graduate Institute of Medical Sciences (SGPGIMS), Lucknow, Mahatma Gandhi Institute of Medical Sciences (MGIMS), Wardha, and Jawaharlal Institute of Post Graduate Medical Education & Research (JIPMER), Puducherry. This survey was done as a part of Indian Council of Medical Research (ICMR) sponsored initiative on "Clinico-epidemiologic and molecular characterization of extended-spectrum beta-lactamase (ESBL) producing *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. causing nosocomial and community Infections".

The identities of all strains submitted were reconfirmed by conventional biochemical methods and API (Biomerieux, Craponne, France) system. The isolates were then tested for extended-spectrum β-lactamase (ESBL) production using the screening criteria described by Clinical and Laboratory Standards Institute (CLSI) 2009[14] at the study central monitoring laboratory (BMPLIII) at CMC, Vellore. From this collection (n=909), 312 cefoxitin resistant isolates [(*E. coli* (n=152), *Klebsiella* spp. (n=117) and *Enterobacter* spp. (n= 43)] were further characterized for AmpC expression.

Antimicrobial susceptibility testing (AST): ESBL-producing isolates were confirmed to be cefoxitin (30 µg) resistant by the Kirby Bauer disc diffusion

method as per CLSI 2009[14]. The minimum inhibitory concentrations (MICs) of cephalosporins, with and without clavulanic acid, for ESBL detection and confirmation as well as the MICs of amikacin, levofloxacin, piperacillin-tazobactam, imipenem, meropenem, ertapenem and tigeycycline for all cefoxitin-resistant isolates detected in this study were determined by E test (Biomerieux, Craponne, France) and results interpreted as per manufacturers and CLSI 2009 guidelines[14]. Multidrug resistance (MDR) was defined as resistance to three or more antimicrobial classes.

Combined disc diffusion test: The differences in inhibition zones for cefoxitin (30 µg) discs alone and in combination with (400 µg) of phenyl boronic acid was determined[4]. The zone diameters were similar and reproducible when the procedure was repeated. An increase of >5 mm in zone diameter in the presence of phenyl boronic acid compared with cefoxitin tested alone was considered to be positive for the presence of an AmpC β-lactamase production.

Molecular characterization of Ambler class C (AmpC) resistance determinants: Multiplex PCR was used to detect the most common plasmid mediated AmpC genes *ACC, FOX, MOX, DHA, CIT* and *EBC* reported in literature using protocol previously reported[4,15].

Preparation of template DNA - A single colony of each organism was inoculated from a Mac Conkey agar plate into 5 ml of nutrient broth (Becton Dickinson, Maryland, USA) and incubated for 16-18 h at 37°C. Cells from 1.5 ml of the overnight culture were harvested by centrifugation at 17,310 *g* for 5 min. After the supernatant was decanted, the pellet was resuspended in 500 µl of distilled water. The cells were lysed by heating at 95°C for 10 min, and cellular debris was removed by centrifugation at 17,310 *g* for 5 min. Supernatant (2 µl) was used as the DNA template source for amplification.

PCR was performed with a final volume of 25 µl in 0.2-ml thin-walled tubes. The primers used for PCR amplification are listed in Table I. Each reaction contained 20 mM Tris-HCl (*p*H 8.4); 50 mM KCl; 0.2 mM each deoxynucleoside triphosphate; 1.5 mM $MgCl_2$; 0.6 mM primers MOXMF, MOXMR, CITMF, CITMR, DHAMF, and DHAMR; 0.5 mM primers ACCMF, ACCMR, EBCMF, and EBCMR; 0.4 mM primers FOXMF and FOXMR[4]; and 1.25 U of *Taq* DNA polymerase (Life Technologies, Rockville, USA). Template DNA (2 µl) was added to 23 µl of the master mixture. The PCR programme consisted of an initial denaturation step at 95°C for 2 min, followed by 30 cycles of DNA denaturation at 94°C for 45 sec, primer annealing at 62°C for 45 sec, and primer extension at 72°C for 1 min. After the last cycle, a final extension step at 72°C for 5 min was added. PCR product (15 µl) was analyzed by gel electrophoresis with 2 per cent agarose (USB Corporation, Cleveland, USA.). Gels were stained with ethidium bromide at 5 µg/ml and visualized by UV transillumination. A 100-bp DNA ladder (Fermentas International Inc. Burlington, Canada) was used as a molecular ladder. Negative controls were PCR mix with water in place of template DNA.

Control strains: *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853, and *E. coli*

Table I. Primers used for characterization of AmpC β-lactamases

Target gene	Primers	Sequences (5' to 3')	Amplicon size (bp)
MOX-1, MOX-2, CMY-1, CMY-8 to *CMY-11*	MOXMF MOXMR	GCT GCT CAA GGA GCA CAG GAT CAC ATT GAC ATA GGT GTG GTG C	520
LAT-1 to *LAT-4, CMY-2* to *CMY-7, BIL-1*	CITMF CITMR	TGG CCA GAA CTG ACA GGC AAA TTT CTC CTG AAC GTG GCT GGC	462
DHA-1, DHA-2	DHAMF DHAMR	AAC TTT CAC AGG TGT GCT GGG T CCG TAC GCA TAC TGG CTT TGC	405
ACC	ACCMF ACCMR	AAC AGC CTC AGC AGC CGG TTA TTC GCC GCA ATC ATC CCT AGC	346
MIR-1T ACT-1	EBCMF EBCMR	TCG GTA AAG CCG ATG TTG CGG CTT CCA CTG CGG CTG CCA GTT	302
FOX-1 to *FOX-5b*	FOXMF FOXMR	AAC ATG GGG TAT CAG GGA GAT G CAA AGC GCG TAA CCG GAT TGG	190

Cephamycins (CMY), Cefoxitin (FOX), and Moxalactam (MOX) or Latamoxef (LAT), AmpC type (ACT) or Ambler class C (ACC), Miriam Hospital in Providence, R.I. (MIR-1) or Dhahran Hospital in Saudi Arabia (DHA), BIL-1 named after the patient (Bilal).
Source of primers: Ref. 4

Table II. Antimicrobial susceptibility profile of 312 cefoxitin resistant study isolates

Antimicrobial agent tested	Cefoxitin resistant, AmpC negative (n=198)		Cefoxitin resistant, AmpC positive (n=114)			
			PCR positive (n=48)		PCR negative (n=66)	
	$MIC_{50/}$ MIC_{90} (µg/ml)	%Sus /Res	$MIC_{50/}$ MIC_{90} (µg/ml)	%Sus /Res	$MIC_{50/}$ MIC_{90} (µg/ml)	%Sus /Res
Amikacin	12/>256	56.6/43.4	4/>256	83.3/16.7	4/>256	86.4/13.6
Levofloxacin	24/>32	19.2/80.8	24/>32	14.6/85.4	24/>32	18.2/81.8
Imipenem	0.25/2	91/9.1	0.19/0.38	98/2.1	0.19/4	100/0
Meropenem	0.064/2	92/ 8.1	0.049/0.25	100/0	0.064/0.47	100/0
Ertapenem	0.25/6	78.8/21.2	0.19/1	98/2.1	0.19/2	92.4/7.6
Tigecycline	0.25/1.5	98/2	0.25/1.5	100/0	0.38/1.5	99/1.5
Pip-Taz	12/>256	60.1/39.9	6/>256	79.2/21	6/>256	72.7/27.3

Sus, susceptible; Res, resistant; Pip-Taz, piperacillin-tazobactam

ATCC 25922 were used to quality check the media, biochemical tests and susceptibility testing. ATCC standard strains were used to quality check E test and test ranges interpreted as per manufacturers, CLSI 2009 guidelines[14]. Amp C genotype standard strains A7 (ACC), A9 (CMY-2), and PMG252 (FOX-5) (provided by Dr George A Jacoby, Lahey Clinic, Burlington, Massachusetts, USA), and clinical isolate ADB05 (DHA), ADB42 (EBC) from our laboratory were used as PCR control strains.

Results

Overall, among 909 Gram-negative isolates, 312 were deemed cefoxitin resistant by Kirby Bauer disc diffusion test. This included *E. coli* (n=152), *Klebsiella* spp. (n=117) and *Enterobacter* spp. (n=43).

Among 312 cefoxitin resistant isolates, AmpC phenotype was confirmed by the combined disc diffusion testing in 36.5 per cent (n=114), in the remaining (n=198) it was not detectable. The overall occurrence of Amp C in the study was determined to be 12.5 per cent. Among the 312 cefoxitin resistant isolates, highest susceptibility to tigecycline (99%) was seen followed by imipenem, meropenem (97%), ertapenem (89%), amikacin (85%), and piperacillin-tazobactam (74.6%), resistance to levofloxacin was high (82%). There were no significant differences in susceptibility as well as MIC_{50} and MIC_{90} values (Table II) among the AmpC producers (n=114) and Amp C negative isolates (n=198). A very high proportion (n=92%) of the AmpC phenotypes was also found to be ESBL producers with a significant number of them (n=81.7%) showing multidrug resistance.

Among 114 isolates of Amp C producers, 48 could be assigned a genotype, this included CIT- FOX (n=25), EBC (n=10), FOX (n = 4), CIT (n=3), and EBC-ACC (n=2), one each of DHA, EBC-DHA, FOX –DHA and FOX-EBC-DHA.

Isolates with plasmid mediated AmpC were recovered from patients with urinary tract (n=21), skin and soft tissue (n=17) and blood stream infection (n=10). Of these, 79.4 per cent were classified to be nosocomial in origin. The phenylboronic acid-cefoxitin disc tests showed corresponding sensitivity of 72.9 per cent, specificity of 45.4 per cent, positive predict value of 49.2 per cent, and negative predict value of 69.7 per cent when compared with PCR. Overall, presence of common plasmid mediated AmpC genotypes among Gram-negative isolates was low (5.2%). In our study, CIT- FOX (21.9%) and EBC Amp C (8.7%) genotypes were predominant. CIT-FOX like enzymes were common in *E.coli* (43.7%) and EBC like enzymes in *K. pneumoniae* (16.6%).

Discussion

The prevalence of plasmid AmpC-mediated resistance in India is not known, due to the limited number of surveillance studies seeking clinical strains producing AmpC β-lactamases and the difficulty that laboratories have in accurately detecting this resistance mechanism. The present study showed plasmid mediated AmpC β lactamases in 12.5 per cent isolates, with commonly reported genotypes seen among 5.2 per cent of them.

Plasmid AmpC β-lactamases have differential activity on β-lactamases inhibitors, *E.coli* derived enzymes have shown to exhibit resistance to inhibitor combinations with possible exception of piperacillin-tazobactam[1]. In previous Indian studies, cefoxitin resistant strains were tested for the production of

AmpC β lactamases by three dimensional extract methods[16,17]. A recent Indian study has recommended use of piperacillin and piperacillin-tazobactam discs for AmpC screening[18]. Cefotetan with phenyl boronic acid has also been used to detect AmpC especially MOX-1, FOX-1, ACT-1 producing isolates[19]. Detecting plasmid mediated AmpC with co-existing ESBL is very challenging. Given these difficulties in detecting plasmid mediated AmpC β-lactamases, their prevalence is currently being underestimated.

AmpC β-lactamases also have differential activity on substrates. *E.coli* with ACC−1 can be resistant to ceftazidime but not to cefotaxime or cefotetan while an isolate with DHA-2 may show intermediate resistance to cefoxitin but susceptible to cefotaxime or ceftazidime[1]. In our study, we could not discern such findings, further analysis of isolates including cephamycin hydrolysis assay is necessary to verify these effects.

In the study, sizeable number of cefoxitin resistant isolates were not positive for AmpC production by the disc potentiation test or PCR, this warrants further investigation into the other mechanisms of resistance and their laboratory detection. Clinical isolates rarely express more than one plasmid-mediated AmpC β-lactamases. Two reasons could explain this observation. First, the inability of current phenotypic tests to accurately detect the type of transferable AmpC β-lactamase does not allow for the differentiation of multiple AmpC enzymes. Second, it is possible that there is a limit to the amount of AmpC β-lactamase that a bacterial cell can accommodate and still be a viable pathogen[20]. A single type of test will not be able to accurately characterize the resistance mechanisms in these complex organisms. Although automated systems are available for susceptibility testing, the accuracy of these are inadequate for organisms expressing plasmid-mediated AmpC β-lactamases alone or in combinations with ESBLs[21-23].

The use of cefoxitin resistance as a screening agent/ marker for AmpC production is quite reliable with a good negative predictive value as found in our study. The use of phenylboronic acid in combination with cefoxitin as a phenotypic screening method may be a better tool for laboratory diagnosis and confirmation of AmpC producing Gram-negative bacteria. The disc potentiation test reliably detected AmpC β-lactamase when compared against the PCR in the present study. Clinical laboratories interested in distinguishing AmpC mediated resistance from other β-lactamase resistance mechanisms 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 plasmid-mediated AmpC β-lactamases genes in Gram-negative bacteria.

In the present study, MDR among AmpC positive study isolates was high suggesting plasmid mediated spread. Current therapeutic options include use of cefepime or carbapenems[7], however, the high co-carriage of ESBL and AmpC in this study and the fact that majority of these were nosocomial in origin is a cause for concern. This study findings indicate the necessity for continued surveillance of mechanisms of resistance among nosocomial pathogens and evolving preventive measures aimed at reducing their spread.

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