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Research Article

IDENTIFICATION OF AMPC B-LACTAMASE-PRODUCING CLINICAL ISOLATES OF *ESCHERICHIA COLI*

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ABSTRACT

Objective: Indiscriminate use of β-lactam antibiotics has resulted in the emergence of β-lactamase enzymes. AmpC β-lactamases, in particular, confer resistance to penicillin, first-, second-, and third-generation cephalosporins as well as monobactams and are responsible for antibiotic resistance in nosocomial pathogens. Therefore, this study was undertaken to screen nosocomial *Escherichia coli* isolates for the presence and characterization of AmpC β-lactamases. The study also envisaged on the detection of inducible AmpC β-lactamases and extended-spectrum β-lactamases (ESBLs) in AmpC β-lactamase-producing *E. coli*.

Methods: A total of 102 clinical isolates of *E. coli,* were subjected to cefoxitin screening, and screen-positive isolates were further subjected to inhibitor-based detection method, phenotypic confirmatory test, disc antagonism test, polymerase chain reaction (PCR), and isoelectric focusing (IEF).

Results: In this study, 33% of *E. coli* were resistant to cefoxitin, of which 35% were found to be positive for AmpC β-lactamase by inhibitor-based phenotypic test. Of the AmpC-positive isolates, 83% were positive for ESBLs, whereas 25% were producing inducible AmpC β-lactamases. PCR and IEF showed CIT and EBC types of AmpC β-lactamases present in the tested isolates.

Conclusion: Our study showed the presence of inducible AmpC enzymes and ESBLs in *E. coli* isolates and PCR identified more isolates to be AmpC producers.

Key words: AmpC β-lactamases, *Escherichia coli*, Extended-spectrum β-lactamases, Inhibitor (Boronic acid)-based test, Isoelectric focusing.

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INTRODUCTION

There has been an implausible rise in the incidence of antibiotic resistance, more importantly to cephalosporins in bacterial pathogens [1,2]. Of the various resistance mechanisms, production of β-lactamases is the most widespread. AmpC β-lactamases are clinically significant as they confer resistance to a broad spectrum of antibiotics such as penicillin, first-, second-, and third-generation cephalosporins and monobactams such as aztreonam and are not inhibited by commercially available β-lactamase inhibitors [3,4]. *Escherichia coli* is a commensal and opportunistic pathogen which can be responsible for intestinal as well as urinary tract infections, and hence, is targets for antibiotic therapies [5]. This can lead, in some cases to a high degree of antibiotic resistance. AmpC β-lactamases are encoded by *amp*C genes which can be chromosomally or plasmid mediated [6]. In *E. coli*, *amp*C is expressed constitutively, but at low level, mutations in the *amp*C promoter/attenuator region can result in constitutive overexpression [7]. Plasmid-mediated *amp*C genes are also reported in *E. coli,* which are expressed constitutively in high levels and are also induced by β-lactams such as ACT-1 β-lactamases [6]. Hence, plasmid-mediated AmpC β-lactamases have considerable clinical significance [8]. Moreover, clinical isolates possessing plasmids encoding AmpC enzymes often are resistant to multiple antibiotics hence, leaving few therapeutic options.

Taking into consideration the significance of AmpC β-lactamases and coexistence of extended-spectrum β-lactamases (ESBLs) in AmpC β-lactamase-producing isolates in clinical world, this study was undertaken to identify and characterize different types of AmpC β-lactamases in *E. coli*. The present study compared different phenotypic methods with a genotypic method polymerase chain reaction (PCR) to detect AmpC β-lactamases in clinical isolates of *E. coli*.

METHODS

Bacterial isolates

A total of 102 isolates of *E. coli* recovered from a variety of clinical specimens were collected from two different hospitals of Delhi during the period from April 2003 to December 2005. All the isolates were identified by standard biochemical methods. *E. coli* ATCC 25922 was included as a negative control in all the tests described, whereas *E. coli* C600 pMG230 and *Klebsiella pneumoniae* 48188 were used as positive controls in all the experiments.

Screening for AmpC production

The isolates were tested for antimicrobial susceptibility to cefoxitin by Kirby-Bauer disc diffusion method [9]. The inhibition zone sizes were interpreted as per the CLSI (formerly NCCLS) guidelines [10]. All the isolates with an inhibition zone diameter of <18 mm were selected.

Detection of AmpC β-lactamases

The screened positive isolates were tested by an inhibitor (boronic acid)-based detection method to detect AmpC-producing *E. coli* [11]. Briefly, a stock solution of boronic acid was prepared by dissolving 120 mg of phenylboronic acid (Sigma-Aldrich, St. Louis, USA) in 3 ml dimethyl sulfoxide, to which 3 ml of sterile distilled water was added. Discs were prepared by dispensing 20 µl of the stock solution on 30 µg of cefotetan discs. These were dried for 30 min and used immediately or stored in airtight vials with a desiccant at 4°C. This test was performed by inoculating Mueller-Hinton Agar (MHA) plate with test isolates by the standard disc diffusion method (CLSI/NCCLS) and placing a disc containing 30 µg of cefotetan along with a disc containing 30 µg of cefotetan and 400 µg of boronic acid. The inoculated plates were incubated overnight at 37°C. Organism showing an increase of 5 mm of zone diameter around the disc containing cefotetan and boronic acid as

compared to that of zone diameter around the disc of cefotetan alone was considered as AmpC producer.

Detection of inducible AmpC β-lactamases

Disc antagonism test (DAT) was carried out as described by Yan *et al*., 2002, [12]. Briefly, a disc of cefoxitin (30 µg) was kept in the center of the inoculated (test isolate) MHA plate and discs of ceftazidime (CAZ), cefpodoxime, and cefotaxime (CTX) were placed around it in a way that each of these was 15 mm apart. The plates were incubated overnight at 37°C. Blunting of the zone of inhibition around any of the cephalosporin discs adjacent to the cefoxitin disc was considered as a positive result.

Phenotypic confirmatory test (PCT) for detection of ESBLs

This test was performed by the disc diffusion technique as recommended by CLSI (NCCLS). A ≥5 mm increase in the zone diameter for either CAZ or CTX tested in combination with clavulanic acid (CLA), versus its zone when tested alone, confirmed an ESBL-producing organism.

All the media and antibiotic discs used for phenotypic tests were procured from Becton, Dickinson & Co., USA.

DNA extraction

A few (2-3) colonies of the test isolates were inoculated in 5 ml Luria Bertani broth (Becton, Dickinson & Co., USA) and incubated overnight at 37°C. Boiled lysate of each of the cultures was prepared by boiling the culture at 100°C for 10 min and collecting the supernatant after centrifugation at 9838× g for 5 min. The supernatant was used as a source of template DNA.

Detection of ampC genes by multiplex PCR

Detection of plasmid-mediated AmpC β-lactamase genes (*amp*C) in all the screened positive isolates was carried out by PCR using the method described by Pérez-Pérez and Hanson 2002, [13]. Family-specific primers, as shown in Table 1, were used to detect different families/types of AmpC β-lactamases in the isolates. Each 50 µl PCR mixture contained 0.2 mM of each dNTPs (Sigma-Aldrich Chemical Pvt. Ltd, Bengaluru, India), 0.5 µM of forward and reverse primers (Sigma-Aldrich Chemical Pvt. Ltd, Bengaluru, India), 1X reaction buffer (New England Biolabs Inc., USA) with 2 mM of $MgCl_2$ (New England Biolabs Inc., USA), 1.25 U of Taq polymerase (New England Biolabs Inc., USA), and 2 µl of DNA. Nuclease-free water was used in all the reactions and as a negative control. PCR was carried out in a PTC-100™ Programmable thermal cycler (MJ Research Inc.) using the following conditions: 94°C for 3 min, 94°C for 30 s, 64°C for 30 s, and 72°C for 1 min for 30 cycles followed by a final extension at 72°C for 7 min. PCR products were visualized on a 2% ultrapure agarose (Sigma-Aldrich, St. Louis, USA) gel stained with ethidium bromide (Sigma-Aldrich, St. Louis, USA, 10 mgL^{-1}).

Nucleotide sequence analysis of ampC genes

Nucleotide sequencing of PCR amplicons was undertaken on both strands using respective PCR-specific primers (Table 1) by dideoxychain termination method using the services of the commercial vendor (Link Biotech, India). Sequence analysis and comparisons were performed using nucleotide BLAST, and sequences were compared against the database of *amp*C genes present at the NCBI server (http://www.ncbi.nlm.nih.gov).

Table 1: List of family-specific primers used for amplification of *ampC* genes

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

Detection and characterization of AmpC β-lactamases by isoelectric focusing (IEF)

Characterization of AmpC β-lactamases was carried out by IEF, following the procedure of Matthew *et al*., 1975, [14] with few modifications. Briefly, crude enzyme extract from test isolates was prepared by subjecting the cell pellet from overnight grown cultures to 20 cycles of freezing at −20°C for 30 min and thawing at 37°C for 15 min. Nitrocefin sensitivity test was performed by loading 50 µl of nitrocefin solution and 10 µl of crude extract of the test organism into a 96-well microtiter plate. The rate of color change was graded on a scale of 5-1. For the score of 5, 1 µl of crude enzyme extract, and for a score of 1, 20 µl of crude enzyme extract were loaded on commercially prepared polyacrylamide gels with a pH range of 3.5-10. Gel electrophoresis was carried out using on a LKB Bromma 2117 Multiphor II electrophoresis unit (Ampholine PAG plate; GE Healthcare Uppsala, Sweden). The gel was run at 1500V, 50mA, and 30W for 90 min. The bands were analyzed by placing a filter paper soaked in nitrocefin and placing it over the gel for 10 min.

RESULTS

Detection of β-lactamases in test isolates

Of the 102 isolates of *E. coli* screened for AmpC β-lactamases production, 34 were identified as screen positive. Of these screen-positive isolates, 12 were identified as AmpC producers by inhibitor (boronic acid)-based test (EC 1, 2, 3, 4, 10, 11, 19, 20, 22, 24, 26, and 29). Of these 12 AmpC producers, 3 isolates, namely, EC 1, 19, and 22 were found positive by DAT suspecting to produce inducible AmpC β-lactamases and 10 isolates (EC 2, 3 ,4, 10, 11, 20, 22, 24, 26, and 29) were found to coproduce ESBLs (Tables 2 and 3).

Detection of *ampC* genes

All the screened positive isolates were subjected to multiplex PCR. Of the 34 screened positive isolates of *E. coli*, 16 were found to show *ampC* genes of amplified products of 462 bp (CIT type) and 302 bp (EBC), respectively (Fig. 1). Of these 16 PCR-positive isolates, 13 harbored *amp*C genes of CIT family (EC 2, 3, 4, 10, 11, 14, 15, 20, 24, 26, 29, 32, and 34) and 3 (EC 1, 19, and 22) of EBC family (Table 4). None of the tested isolates harbored genes for MOX, ACC, DHA, and FOX families. CIT-type *amp*C gene was found to be predominant in the PCR-positive isolates (Table 4). Nucleotide sequencing of the CIT family amplicons was found to have 100% homology to CMY-2 and EBC family amplicons to have 100% homology to ACT-1-type AmpC β-lactamases. PCR detected four additional isolates: EC 14, 15, 32, and 34 to produce AmpC β-lactamases as compared to phenotypic test, and all these isolates produced CIT-type AmpC enzyme (Table 3).

Characterization of AmpC β-lactamases by IEF

All the screened positive isolates were subjected to IEF. Of the 34 screened positive *E. coli*, bands of pI values of >8.4 corresponding to CIT or MOX types were detected in 6 isolates (EC 2, 24, 26, 30, 31, and

Table 2: Number of *E. coli* isolates positive for β-lactamases by various phenotypic methods

Detection methods	Number of isolates positive (%)
Cefoxitin screening n=102	34 (33)
Inhibitor (boronic acid)-based detection n=34	12 (35)
DAT n=12	3 (25)
PCT (PAT) n=12	10 (83)

n: Total number of isolates tested, PCT: Phenotypic confirmatory test, DAT: Disc antagonism test, *E. coli: Escherichia coli*

Table 3: Representation of different clinical isolates of *E. coli* found positive by different detection method

Isolates	Inhibitor-based detection	DAT	PCT	PCR	IEF
EC1					
EC2					
EC3					
EC4					
EC5					
EC6					
EC7					
EC8					
EC9					
EC10					
EC11					
EC12					
EC13					
EC14					
EC15					
EC16					
EC17					
EC18					
EC19					
EC20					
EC21					
EC22					
EC23					
EC24					
EC25					
EC26					
EC27					
EC28					
EC29					
EC30					
EC31					
EC32					
EC33					
EC34					

Red highlight depicts isolates positive for β-lactamases by respective methodologies, PCR: Polymerase chain reaction, PCT: Phenotypic confirmatory test, IEF: Isoelectric focusing, DAT: Disc antagonism test, *E. coli: Escherichia coli*

Table 4: Detection and characterization of AmpC β-lactamases by PCR

AmpC β-lactamase families	Number of isolates positive (%)
CIT	13 (81.25)
EBC	3 (18.75)
MOX	-
DHA	-
ACC	-
FOX	-
Total	16

Total number of isolates tested =34, PCR: Polymerase chain reaction

34) and bands of pI 8.4 corresponding to EBC type in 3 (EC 1, 19, and 22). Bands of pI values ranging between 6.3 and 7.2 corresponding to either FOX or DHA types in 3 (EC 5, 6, and 21) and bands of pI 7.6 corresponding to ACC type were detected in 4 isolates (EC 8, 23, 25, and 33) (Fig. 2 and Tables 3 and 5). Interestingly, in 12/34 isolates of *E. coli*, bands of pI 5.4 could be detected, which did not correspond to any of the known AmpC types (data not shown). In addition, 6 of the isolates showed the presence of multiple β-lactamase bands (data not shown).

DISCUSSION

Indiscriminate and large-scale use of β-lactam antibiotics has led to the emergence of β-lactamase enzymes such as ESBLs and AmpC β- lactamases in a variety of bacterial pathogens and are responsible for high-level resistance to these antibiotics [15-20]. Varying rates of incidence ranging from as low as 1.6% to as high as 60% of AmpC-

Table 5: Detection and characterization of AmpC β-lactamases by IEF

pI values of bands detected	Corresponding presumptive AmpC types	Number of isolates positive (%)
>8.4	CIT, MOX	6 (17.6)
8.4	EBC	3 (8)
7.6	ACC	4 (11)
6.3-7.2	FOX, DHA	3 (8)
5.4	-	12 (35.2)

Total number of isolates tested =34, IEF: Isoelectric focusing

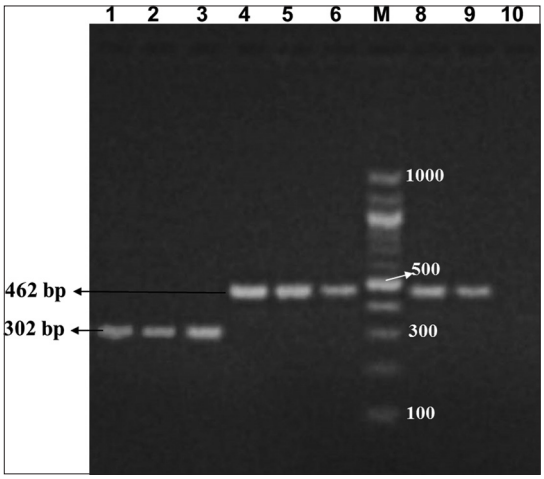

Fig. 1: Identification of AmpC β-lactamase types by polymerase chain reaction; Lanes 1: Positive control for EBC type: 2 and 3: Positive isolates of *Escherichia coli* for EBC type; 4-9: Positive isolates of *E. coli* for CIT type, M: 100 bp molecular weight marker; 10: Water control as negative

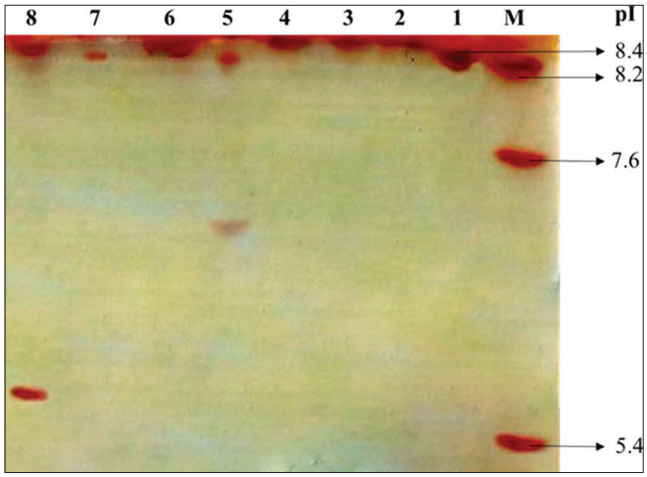

Fig. 2: Example of isoelectric focusing gel electrophoresis carried out in this study. Lanes M: Standard marker of different pI values; 1: Positive control for EBC type; 2, 3, 4, 6, and 8: Isolates positive for CIT/MOX types; lane 8 shows the presence of another band of a different pI value; 5 and 7: Isolates positive for EBC types, lane 5 also shows the presence of a band of different pI value

producing *E. coli* isolates have been reported from various parts of the world [11,21-24]. Studies from different geographical areas in India have reported 6% to 41% of *E. coli* isolates to be AmpC producers [22,25-28]. Our study showed similar results (35% by phenotypic test) to some of the researchers reporting a high incidence of AmpC β-lactamases in *E. coli*, indicating a rising trend of resistance, which should be a cause of concern.

Amoxicillin-CLA combination is usually given in treating infection caused by β-lactamase-producing isolates because CLA is known to be an active inhibitor of ESBLs. Since CLA can act as an inducer for AmpC β-lactamases, it becomes imperative to check for the presence of inducible AmpC β-lactamases in the clinical isolates. Studies have reported the presence of inducible AmpC β-lactamases in 4-21% of *E. coli* isolates [25,29]. Our study showed similar results with 16.6% of the AmpC-positive isolates to be inducible; however, more number of isolates need to be tested to show an increasing trend. Hence, testing for the presence of inducible AmpC producers becomes important, as there can be a therapeutic failure if antibiotics inducing the enzymes are administered for treating the infection empirically.

A multiplex PCR which distinguished plasmid-mediated AmpC enzymes from chromosomally mediated and also differentiated between six different families of plasmid-mediated AmpC β-lactamases was used in this study. A number of workers have used PCR to detect the presence of *ampC* genes *E. coli* and have reported PCR to be more sensitive than phenotypic methods to detect AmpC-producing isolates [4,30-32]. Our study showed the presence of *ampC* gene for plasmid-mediated AmpC enzymes in 47% of the screen-positive isolates of *E. coli* and was found to detect four additional AmpC producers than inhibitor-based method. In the present study, a high percentage (81.2%) of the PCR-positive isolates of *E. coli* were shown to produce CIT type of AmpC β-lactamases, followed by 18.75% producing EBC type. Our study corroborates the findings of other studies, showing a high percentage (88%) of *E. coli* isolates to produce CIT type of AmpC enzymes [28,33,34]. However, there are studies which reported a lower prevalence, 11%, 34%, and 56%, respectively, of the *E. coli* isolates to be producing CIT-type AmpC β-lactamases [4,35,36]. The presence of EBC family or ACT-1 subtype of AmpC enzymes in *E. coli* has been reported in many studies; however, EBC-type AmpC β-lactamases was found to be more prevalent in *Klebsiella* spp. [37-40]. In the present study, we found ~19% of the inducible AmpC-producing *E. coli* isolates to be of EBC (ACT-1) type. In our study, inducible AmpC β-lactamases were detected by DAT, IEF as well as PCR.

The inhibitor-based phenotypic test detected less number of isolates to be AmpC positive as compared to the PCR, and this suggests that PCR should be used as the gold standard method for identification of beta-lactamases. However, more number of isolates need to be tested to confirm this.

Detection and characterization of β-lactamases employing IEF were for the first time carried out by Matthew *et al*. in 1975 [14]. Subsequently, IEF was used for characterizing AmpC β-lactamases in a number of bacterial pathogens [12,41-43]. In the present study, β-lactamase bands of pI values >8.4, corresponding to CIT type, bands of pI value 8.4 corresponding to EBC type, bands of pI values ranging between 6.3-7.2 corresponding to FOX/DHA, and bands of pI value 7.6 corresponding to ACC type AmpC enzymes were observed. These were further confirmed by PCR to be the respective AmpC enzymes except ACC and FOX or DHA types. According to our PCR results, none of the *E. coli* isolates harbored *amp*C genes for ACC and DHA families. In comparison to PCR, IEF detected less isolates to produce CIT type but extraisolates to produce ACC and FOX-/DHA-type AmpC β-lactamases. Moreover, bands of pI value 5.4 were detected which did not correspond to any of the known AmpC β-lactamases. This suggests that there may be a possibility that these pI bands correspond to some ESBLs and that some of the tested isolates coproduced ESBLs. It was found that 83% of the AmpC-producing isolates in this study were also producing ESBLs. Many studies have shown the presence of ESBLs along with AmpC enzymes which also showed bands of different pI values [24,27,44-47]. In this study, there was one isolate, EC 22, which was found to be positive for inducible AmpC β-lactamases, coproduced ESBL and was found to harbor genes for ACT-1 subtype of AmpC β-lactamase. This coexistence of multiple enzymes in clinical isolates may restrict the therapeutic options available for treating cases infected with such pathogens.

CONCLUSION

Our findings may suggest that there is a need to initiate steps to rationalize the use of the β-lactams and also to have a more vigorous surveillance for detection of such resistance in clinical isolates both at the hospital as well as at the community level. However, a larger set of *E. coli* isolates along with other nosocomial pathogens need to be tested to have more substantiate data. This constitutes the part of our future studies.

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