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Occurrence of Plasmid Mediated AmpC β-lactamase genes and their types among the clinical isolates of *Escherichia coli* from a Tertiary Care Hospital of Punjab (North India)

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Abstract: AmpC β- lactamases, which are often plasmid mediated, confer resistance to a wide variety of β-lactam and β-lactam inhibitor combinations. These are commonly detected phenotypically but the studies demonstrating their genotypic characterization are lacking. Hence, the present study was designed to determine the occurrence of plasmid mediated AmpC β-lactamase genes and their types among the clinical isolates of *Escherichia coli*. Two hundred and fifty consecutive, non-duplicate clinical isolates of *E.coli* recovered over a period of one year (January 2014-December 2014) were screened for AmpC production by cefoxitin resistance. Boronic acid disc potentiation (DDST) and modified 3 dimensional tests (M3DT) were used for phenotypic detection of AmpC production. AmpC genotypes ACC, FOX, MOX, DHA, CIT and EBC were detected by multiplex PCR. Among the 250 isolates, 90 (36%) were found to be cefoxitin resistant. DDST test was positive in 27 (30%) while M3DT in 29 (32.2%) of cefoxitin resistance (90) strains. By PCR, the plasmid encoded AmpC genes were detected in 27.7% (25/90) isolates which gave an overall prevalence of 10% (25/250). The gene detected in all the isolates was CIT only. Multidrug resistance (MDR) was noticed among 24% (6/25) AmpC producing strains. A high percentage of plasmid-encoded AmpC enzymes and MDR among them suggests plasmid mediated spread of drug resistance in *E. coli* in the present study. The exact detection of plasmid mediated AmpC β- lactamases (PMABLs) by using genotypic tests along with the phenotypic tests would be helpful in rational antimicrobial therapy, epidemiology and infection control.

Keywords: *E.coli*; AmpC β- lactamase; multiplex PCR; multidrug resistance; CIT gene

INTRODUCTION

Escherichia coli (*E. coli*) are one of the commonest gram negative opportunistic pathogen of nosocomial infections. The predominant mechanism of resistance in this organism is the synthesis of β-lactamases [1,2]. AmpC β-lactamases are clinically important cephalosporinases which confer resistance to a wide variety of β-lactam antibiotics including 7-α-methoxycephalosporins, oxyiminocephalosporins and monobactams and are not inhibited by β-lactamase inhibitors such as clavulanic acid [3]. It is presumed that initially these enzymes were chromosomally mediated but had disseminated to plasmids. Thus, the plasmid mediated AmpC genes have been derived from inducible chromosomal genes that have become mobilized. Due to their mobility, they have the capability to emerge in genera or species of different organisms resulting in substantial threat of drug resistance among the gram negative bacteria [4]. Although reported increasingly, the true rate of occurrence of AmpC β lactamases in most of the countries is still unknown. Various studies from India have reported that the prevalence of the AmpC β lactamase producers among gram negative bacteria varies between 3.3% and 47.8% but in most of these studies AmpC production has not been examined at the molecular level [5-9]. In view of the paucity of molecular studies of AmpC β- lactamase production by *E. coli*, the present study was undertaken to determine the occurrence of PMABL gene types among the isolates of *E. coli* from a tertiary care hospital of Malwa region of Punjab (North India).

MATERIAL AND METHODS

A total of 250 consecutive, non-duplicate isolates of *E.coli* obtained from various clinical specimens such as urine, pus, blood and body fluids received in the Department of Microbiology of a tertiary care hospital of Punjab, over a period of one year (January, 2014 to December, 2014) were included in the study. All the isolates were identified as per the standard bacteriological procedures [10]. The

antibiogram of the isolates to various antibiotics such as ampicillin (2µg), ceftazidime (30µg), cefotaxime (30µg), ceftriaxone (30µg), cefoxitin (30µg), amikacin (30µg), ciprofloxacin (5µg), imipenem (10µg), amoxicillin-clavulanic acid (20 + 10µg), was determined by the Kirby-Bauer's disk diffusion method according to Clinical Laboratory Standards Institute (CLSI) guidelines [11]. *E. coli* ATCC 25922 was used as a quality control strain. The isolates which were resistant to one or more third generation cephalosporins were screened for AmpC β-lactamase production. The isolates which yielded <18 mm zone diameter around cefoxitin disc were taken as putative AmpC producers [12] and were subjected to Boronic acid disc potentiation (DDST) and Modified three dimensional tests (M3DT) for phenotypic confirmation [12,13]. In Boronic acid disc potentiation test, AmpC production was detected by observing an increase in zone of inhibition by ≥ 5mm around cefoxitin- boronic acid disc than that around disc containing cefoxitin alone;[12] while in the Modified three dimensional test isolates which showed clear distortion of zone of inhibition of cefoxitin disc by enhanced growth of surface organism were taken as AmpC producers. Isolates with no distortion were considered as AmpC non-producers [13].

For molecular characterization, all the cefoxitin resistant *E.coli* strains were subjected to multiplex PCR to identify six family specific AmpC genes namely; FOX, MOX, CIT, DHA, EBC and ACC [14]. For this purpose plasmid DNA extraction was done using plasmid DNA extraction kit (Macherey-Nagel). The extracted DNA was amplified using the primers as described by Perez *et al* [15]. (Table 1). The amplified products were analyzed by gel electrophoresis in 2% agarose gel stained with ethidium bromide. (Figure 1)

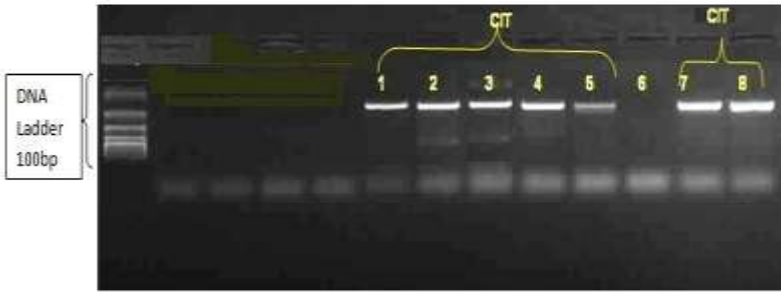

Fig-1: Multiplex PCR showing plasmid-encoded AmpC β-lactamase CIT genes

Table-1: Primers used for characterization of AmpC β-lactamases

Primer	Sequence (5′ to 3 ′, as synthesized)	Amplicon size (bp)
CITMF	TGG CCA GAA CTG ACA GGC AAA	462
CITMR	TTT CTC CTG AAC GTG GCT GGC	
DHAMF	AAC TTT CAC AGG TGT GCT GGG T	405
DHAMR	CCG TAC GCA TAC TGG CTT TGC	
MOXMF	GCT GCT CAA GGA GCA CAG GAT	520
MOXMR	CAC ATT GAC ATA GGT GTG GTG C	
ACCMF	AAC AGC CTC AGC AGC CGG TTA	346
ACCMR	TTC GCC GCA ATC ATC CCT AGC	
EBCMF	TCG GTA AAG CCG ATG TTG CGG	302
EBCMR	CTT CCA CTG CGG CTG CCA GTT	
FOXMF	AAC ATG GGG TAT CAG GGA GAT G	190
FOXMR	CAA AGC GCG TAA CCG GAT TGG	

RESULTS

Out of 250 *E.coli* isolates, 90(36%) showed cefoxitin resistance. The two phenotypic tests i.e. DDST and M3DT confirmed the presence of AmpC production in 27/90 (30%) and 29/90 (32.2%) isolates respectively. In all, 33(36.7%) strains were phenotypically confirmed to be AmpC producers.

Plasmid mediated AmpC β-lactamase (PMABL) genes were detected by multiplex PCR in 25/90 (27.7%) of cefoxitin resistant strains and the genes detected in all these isolates were CIT. No isolate was found to harbour FOX, MOX, DHA, EBC and ACC genes (Figure 1).

The antibiogram of 25 molecularly confirmed AmpC producing strains showed high level resistance against ampicillin (100%), third generation cephalosporins (ceftazidine, cefotaxime and ceftriaxone 100% each) followed by ciprofloxacin (92.1%) and

amoxyclavulanic acid (57.9%). Sensitivity to imipenem and amikacin was 97.3% and 78.9% respectively (Figure 2). Multidrug resistance (MDR) was observed in 6/25 (24%) of PMABL strains.

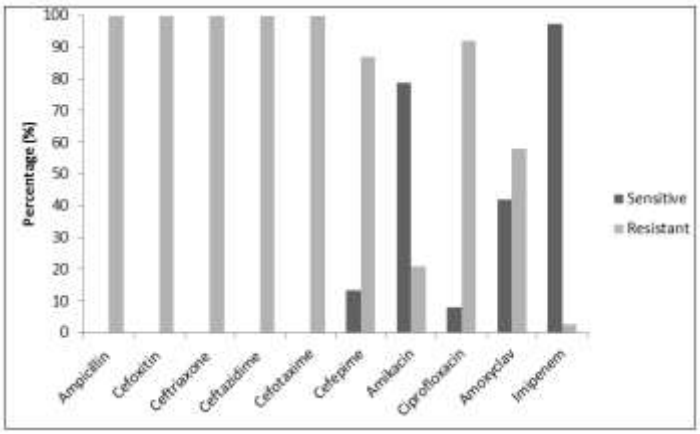

Fig-2: Antibiogram of molecularly confirmed AmpC β-lactamase producing *E coli* isolates

DISCUSSION

AmpC β lactamase mediated resistance constitutes an emerging therapeutic problem in infections caused by *E coli* and other members of family *Enterobacteriaceae*. During the last decade it has been reported from different parts of India (6.97% from New Delhi, 47.8% from Kolkata and 37.5% from Chennai) [5-9, 16]. The present study found it to be 36.7% which corrobrates the findings from Chennai and Kochi, but is higher than some other studies [4-9, 16]. This variation could, in part be, because of lack of CLSI guidelines for phenotypic methods to screen and detect AmpC activity in *Enterobacteriaceae* [11]. The high prevalence in our study could also be due to empirical cephalosporin therapy which is a known mechanism for increasing selective pressure.

In the present study 36% (90/250) of E. coli isolates were labelled as putative AmpC producers on the basis of cefoxitin resistance. This is almost similar to the findings of Manoharan et al who observed cefoxitin resistance in 36.5% of their gram negative isolates and found cefoxitin resistance, a quite reliable marker for screening of AmpC production [9]. However, other authors are of the opinion that cefoxitin resistance does not reliably indicate AmpC production [14]. In the present study, phenotypic tests further used for detection of AmpC production were DDST and M3DT because these are less prone to inter-observer variability and are the most widely used tests. Still, it was observed that Boronic acid disc potentiation test failed to demonstrate AmpC enzyme in 70% (63/90) and modified there dimensional test in 67.7% (61/90) of our isolates. These finding are consistent with the results of other workers [14, 17] and could be because cefoxitin resistance in AmpC non producers might be due to the presence of some other mechanism of resistance such as alteration of outer membrane permeability caused by porin channel mutations [14,17].

As the phenotypic tests alone are not helpful to differentiate between plasmid mediated enzyme producers and the chromosomal hyper producers or porin loss mutants, to know the true number of PMABL producers, all the 90 cefoxitin resistance strains of *E. coli* were subjected to DNA amplification for PMABL coded genes using multiplex PCR; which is considered as a gold standard test to determine the true occurrence of AmpC β lactamases [14]. The plasmid encoded AmpC genes were observed in (25/90) 27.7% isolates which gave an overall prevalence of 10% (25/250) in our study.

A multi-centric study from six geographically different regions of the country reported the presence of these genes in 38.1% of isolates of *E.coli* [9]. A quite low prevalence of 4.6% has also been reported in another study [16]. The 25 PMABL producing strains of our study showed positivity for CIT genotype only. These results are consistent to the finding of Madhavan and Jayalakshmi, 2016 [18]. Other studies from different part of India have reported DHA and MOX genes along with CIT in their *E.coli* isolates [4, 9, 19]. The ACC, EBC and FOX genes have also been reported along with predominant CIT, DHA and MOX genes in some other Indian studies [4, 9]. The presence of CIT gene only among the *E.coli* isolates of our study as well as its predominance in gram negative bacilli in other studies [4, 9, 17, 19] suggests a rapid dissemination of the plasmid mediated CIT gene which is posing a substantial threat with the emergence of multidrug resistance.

The occurrence of multidrug resistance in 24% of PMABL strains of *E.coli* in our tertiary care hospital

though less than that reported by other studies [4, 9], is quite worrisome. In infections caused by these resistant organisms, the therapeutic options are restricted to the use of cefipime or carbapenams only and with the loss of outer membrane porins, these strains become resistant even to carbapenams. Hence, the exact identification of type of AmpC is important for the rational antibiotic therapy to prevent overuse and inappropriate use of antibiotics. The study also emphasizes the need of continued surveillance of mechanisms of resistance. Since phenotypic methods alone may not reflect the true occurrence of PMABL producers, use of genotypic methods need to be employed in the national surveillance studies.

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