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Original Article

PREVALENCE OF AMPC B-LACTAMASES IN CLINICAL ISOLATES OF *E. COLI* FROM A TERTIARY CARE RURAL HOSPITAL

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ABSTRACT

Objective: Organisms over expressing AmpC (Ambler Class C) β-lactamases are of clinical concern because they restrict therapeutic options causing treatment failures and are increasing in occurrence worldwide. So the present study was to undertaken with the aim to know the prevalence of plasmid mediated AmpC and inducible AmpC β-lactamases in clinical isolates of *E. coli* in our tertiary care rural hospital.

Methods: 74 cefoxitin resistant *E. coli* isolates were tested for AmpC production by combined disc diffusion test and disk approximation test.

Results: Out of 74 cefoxitin resistance *E. coli* isolated from various clinical specimen 25(33.78%) showed AmpC β-lactamases production. PMABL was seen in 22(29.73%) and inducible AmpC in 3(4.05%). Among 25 AmpC producing *E. coli*, 8(32%) were from urine, 5(20%) from miscellaneous, 4(16%) from sputum and 12% respectively from stool and Pus and in Blood 2(8%). Age-wise higher distribution of AmpC β-lactamase was in an age group below 1yr (44.44%) and in age group of 20-39yrs (40%). The higher distribution of AmpC β lactamases producer from Medicine, Obgy, ICU(20% respectively) paediatric 16%,surgery 8%, TB 12% and lower from OPD(4%). In our study, multidrug resistance has been observed among the PMABL producing strains. Higher resistance was seen in gentamicin 22(88%), ciprofloxacin 23(92%), ceptazidime 25(100%), cefaclor 25(100%). Whereas PMABL isolates was susceptible to tigecycline (100%), meropenem (92%), amikacin(60%).

Conclusion: The overall prevalence of 10.50% AmpC β-lactamase in *E. coli* and Multidrug resistance is a matter of concern. So identification of AmpC may help in formulating the hospital infection control committee decreasing the selective antibiotic pressure.

Keyword: Cefoxitin resistance, *Escherichia coli,* AmpC β lactamases, Combined disc diffusion test and Disk approximation test.

INTRODUCTION

Gram-negative bacteria pose a therapeutic problem not only in the hospital settings, but also in the community as they have acquired resistance to multiple antibiotics. Organisms over expressing AmpC β-lactamases are of clinical concern because they restrict therapeutic options causing treatment failures and are increasing in occurrence worldwide. AmpC β-lactamases belong to Ambler class C or Group I of Bush's functional classification, they confer resistance to cephalosporins in the oxyimino group (cefotaxime, ceftriaxone, ceftazidime), 7 alpha methoxy cephalosporins (CX) and are not affected by available β-lactamase inhibitors (clavulanate, sulbactam) [1]. Resistance to expanded-spectrum cephalosporins may develop through the expression of chromosomally encoded class C β-lactamases, also known as AmpCβ-lactamases.

These are of two types of Amp C-chromosomally mediated (inducible or constitutive) or plasmid mediated non-inducible [2].

Plasmid mediated AmpC β-lactamases (PMABLs) was first reported in 1988 and 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 Amp C β-lactamases and are typically associated with broad multidrug resistance [3, 4].

The Amp C β-lactamases have been named based on their resistance to cephamycin (CMY), cefoxitin (FOX), moxalactam (MOX), latamoxef (LAT); site of discovery such as Miriam Hospital in Providence (MIR) or Dhahran Hospital in Saudi Arabia (DHA) or name of the source patient, Bilal (BIL). Currently there are 43 CMY alleles, 7 varieties of FOX, 3 varieties to ACT and MOX, 2 varieties of DHA and 4 varieties of ACC, LAT and MIR each [2].

Amp C genes are grouped into six families based on the similarities in the gene sequence and/or origin as CIT (origin Citrobacter freundii), EBC (origin Enterobacter cloacae), DHA (origin Morganella morgannii), ACC (origin Hafnia alvei, FOX (origin unknown) and MOX (origin unknown) [2].

Plasmid-mediated AmpC enzymes have been described from diverse geographic areas, including the United Kingdom, the United States, and Asia [5-8]. In India, prevalence of AmpC β-lactamases in *E coli* has been reported from 3.3% to37.5% [9, 10].

Reduced susceptibility to cefoxitin in the Enterobacteriaceae may be an indicator of AmpC activity, but cefoxitin resistance may also be mediated by alterations to outer membrane permeability [11]. Differentiation 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 producers and carbapenems for the cefoxitin-resistant AmpC producers). Differentiation between them 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 [12].

Detection of AmpC β-lactamases is a challenge to clinical microbiologists. Currently, there are no CLSI-recommended guidelines to detect AmpC β-lactamases [13]. Several phenotypic methods for detection methods of AmpC β-lactamases are described. AmpC screening using disk diffusion, combined disc diffusion test, modified three-dimensional test. But 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 [4].

Coudron *et al.* Used the standard disk diffusion breakpoint for cefoxitin (CX) (zone diameter<18 mm) to screen isolates and used a 3D extract test as a confirmatory test for isolates that harbour AmpC β-lactamases [3]. The detection of plasmid mediated Amp C resistance is important to improve the clinical management of infection and to provide sound epidemiological data [12]. Although reported with increasing frequency the true occurrence in different organisms remains unknown.

So the present study was to undertaken with the aim to know the prevalence of plasmid mediated AmpC and inducible AmpC β-lactamases in clinical isolates of E. coli in our tertiary care rural hospital.

MATERIALS AND METHODS

The prospective study was carried out in the Department of Microbiology, MIMER Medical College, Talegaon Dabhade, Pune from the period of January 2013 to August 2014. A total of 238 non-duplicate clinical isolates of *Escherichia coli* were randomly selected and studied. Sample was processed and isolates were identified by standard laboratory methods [14].

Antibiotic susceptibility testing was done according to CLSI-recommended Kirby–Bauer disk diffusion testing. A total of 74 *Escherichia coli* isolates showing resistance to cefoxitin (inhibition zone<18 mm), a 3rd generation cephalosporins were considered as putative AmpC producers. This isolates were tested for AmpC production by combined disc diffusion test (using cefoxitin (FOX) alone and in combination with BA) and iAmpC by the use of disk approximation test.

Combined disc diffusion test: A lawn of the test organism was made on the Mueller–Hinton agar (MHA) after adjusting the inoculum to 0.5 McFarland units. The cefoxitin discs (30mcg) and cefoxitin (30mcg) discs in combination with (400mcg) of phenyl boronic acid) were placed on MHA and incubated at 35 ^{O}C for 18–24 h in ambient air. 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 [15].

Disks containing boronic acid were prepared as follows: 120 mg of phenylboronic acid (benzeneboronic acid; Sigma-Aldrich, Milwaukee, Wis.) was dissolved in 3 ml of dimethyl sulfoxide. 3 ml of sterile distilled water was added to this solution. Twenty microliters of the stock solution was dispensed onto disks containing cefoxitin (30mcg). Disks were allowed to dry for 30 min and used immediately.

Disk approximation test was used to detect inducible AmpC activity.

Here a disc of 10mcg imipenem, as the inducing substrates and 30mcg ceftazidime disks as the reporter substrate. Disks were placed at a distance of 20 mm Mueller-Hinton agar, and incubated at 35 °C for 16 to 18 h. Any obvious blunting or flattening of the zone of inhibition between the ceftazidime disk and the inducing substrates was interpreted as a positive result for AmpC [16].

The results were statistically analysed by z test.

RESULTS

Out of 74 cefoxitin resistance *Escherichia coli* isolated from various clinical specimen 25(33.78%) showed AmpC β-lactamase production. PMABL was seen in 22(29.73%) and inducible AmpC in 3(4.05%) (table 1).

Table 1: Prevalence of Amp C β-lactamase among the clinical specimen

Specimen	Total no	Cefoxitin resistance *Escherichia coli*	AmpC β-lactamase producer	inducible AmpC
Urine	88	31	6 (19.34%)	2 (6.45%)
Pus	47	6	3 (50%)	0
Miscellaneous	56	25	4 (16%)	1 (4%)
Sputum	14	4	4 (100%)	0
Blood & Fluid	11	3	2 (66.67%)	0
Stool	22	5	3(60%)	0
Total	238	74	22 (29.72%)	3 (4.05%)

The above table depicts higher Prevalence of AmpC β-lactamase was from sputum and lower from urine (19.34%), The prevalence of AmpC β-lactamase production in in our study was higher in female patients 15/43(34.88%) than in males 10/31(32.26%). (table 2) No Statistical significance was noted gender-wise.

Table 2: Age-wise distribution of AmpC β-lactamase

Age	Total no	AmpC β-lactamase producer
1day-1 yr	9	4(44.44%)
1 yr-5yr	4	1(25%)
5-19yr	11	3(27.27%)
20-39 yr	25	10(40%)
40-59 yrs	15	4(26.67%)
60-79 yrs	9	3(33.33%)
80-100yrs.	1	0

In the above table it is noted the distribution of AmpC β-lactamase was higher among the age group below 1yr (44.44%) and in age group of 20-39yrs (40%)

DISCUSSION

Organisms over expressing AmpC β lactamases are a major clinical concern because these are usually resistant to all β lactam drugs except for cefepime, cefpirome and carbapenems. Failure to detect AmpC β lactamase producing strains has contributed to their uncontrolled spread and therapeutic failures. Hence their appearance in a hospital setting should be identified quickly so that appropriate antibiotic use and containment measures can be implemented [17].

In our study out of 238 non-duplicate clinical isolates of *Escherichia coli*, cefoxitin resistant was observed in 74(31.09%) isolates and were thus considered as putative AmpC producers. Similar were the findings of Smitha O. B *et al.* who reported cefoxitin resistant in *E. coli* as 30% and Anand M *et al.* who found among 909 Gram-negative isolates, 312 (34.32%) were deemed cefoxitin resistant by Kirby Bauer disc diffusion test [18, 19]. Higher cefoxitin resistant was observed by Parveen R *et al.* and RM Shoorashetty who in their study reported 77.5% and 45.5% respectively [20, 21].

The use of cefoxitin resistance as a screening agent/marker for AmpC production is quite reliable with a good negative predictive value [3, 19]. But some of the studies has shown that cefoxitin is a poor screening agent for AmpC production because mechanisms other than AmpC such as porin channel mutation may lead to cefoxitin resistance leading to false positive interpretation [22].

Boronic acid (BA) derivatives were reported as reversible inhibitors of AmpC enzymes [23]. These inhibitors have also been incorporated

into disk-based assays, using a variety of combinations of antibiotic substrates and inhibitors. The use of disk approximation tests by Kirby-Bauer testing to detect inducible AmpC activity has also been described, using one antibiotic as an inducing substrate and a second antibiotic as a reporter substrate [24].

For laboratory diagnosis and confirmation of AmpC production, the use of phenylboronic acid in combination with cefoxitin is a better tool for phenotypic screening. The disc potentiation test reliably detected AmpC β-lactamase when compared against the PCR [19].

A recent Indian study has recommended use of piperacillin and piperacillin-tazobactam discs for AmpC screening [25]. Cefotetan with phenyl boronic acid has also been used to detect AmpC especially MOX-1, FOX-1, ACT-1 producing isolates [26].

Song *et al.* reported that the FOX–BA method was 97.7% sensitive for AmpC detection while RM Shoorashetty *et al.* showed it to be only 86.4% [27, 21].

Out of 74 cefoxitin resistance *Escherichia coli, 25*(33.78%) isolate showed AmpC β-lactamase production. PMABL was observed in 22(29.73%) and inducible AmpC in 3(4.05%). Similar were the findings of Anand M *et al.* and Smitha O. B *et al.* who reported Plasmid mediated Amp C phenotype in 36.5% cefoxitin resistant isolates and 24% respectively [19, 18].

Parveen R *et al.* observed the AmpC production in 63.4 % (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 [20]. Whereas in a study by RM Shoorashetty *et al.*, out of 200 clinical isolates of *Enterobacteriaceae*, 14 (7%) isolates showed inducible AmpC (iAmpC) β-lactamases [21].

A 2004 reports from the United States documented 4% of the *Escherichia coli* isolates contained plasmid mediated AmpC type enzymes [7]. Plasmid mediated AmpC was present in 26% of the study isolates, with CMY like enzymes detected predominantly in *E. coli* and DHA like enzymes predominantly in *K. pneumoniae* in a study from Singapore [5]. A study from Switzerland reported the lowest rates of AmpC genes 0.2% [28]. On the contrary, the highest prevalence of AmpC genes in *E. coli* was reported in a Korean surveillance showing 73 % [29].

Geographical variation has been noted in AmpC production in *E. coli* from various parts of the country: 6.97% from north India and eastern part 47.8% [17, 30]. From southern States: studies from Chennai, and 37.5% and 9.2%. (25, 9); and in Karnataka 3.3% of *E. coli* [10].

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 of plasmid-mediated AmpC β-lactamase.

The present study showed the overall prevalence of plasmid mediated AmpC β lactamases in 25/238 isolates (10.50%), comparable to the findings of Anand M *et al.* 12.5% isolates [19].

In specimen-wise distribution of 25 Amp C producing strains of *E. coli*, we noted 8(32%) were from urine, 5(20%) from miscellaneous, 4(16%) from sputum and 12% respectively from stool and Pus and in Blood 2(8%). In a study by Smitha O. B *et al.*, among the total 24 AmpC producing strains of *E. coli*, 12(50%) were from urine specimens, 7(29%) from pus, 3(13%) from sputum, 2(8%) from body fluids [18]. (Chart 1)

The above chart depicts higher distribution of AmpC β lactamases producer from medicine, Obgy, ICU and lower in isolates from OPD

In our study, multidrug resistance (resistance to 3 or more drugs) has been observed among the PMABL producing strains. Amp C producing *E. coli* isolates showed high sensitivity to tigecycline 100%, meropenem 92%, amikacin 60%, whereas Anand M *et al.* observed susceptibility to tigecycline was highest (99%) followed by imipenem, meropenem (97%), ertapenem (89%), amikacin (85%), and piperacillin-tazobactam (74.6%). Levofloxacin resistance was 82% [19].

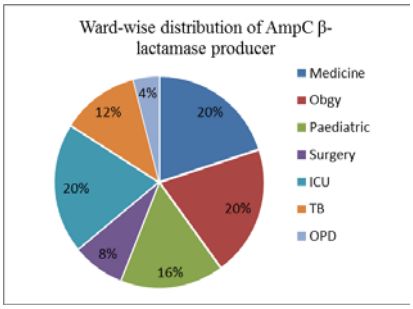

Chart 1: Ward-wise distribution of Amp C β-lactamase

In our study, higher antimicrobial resistance in AmpC producing *E. coli* isolate was seen to gentamicin 22(88%), ciprofloxacin 23(92%), ceptazidime 25(100%), cefaclor 25(100%). Similar was the findings Smitha O. Bagali *et al.*: gentamicin (95.8%), amoxycillin+clavulanate (95.8%), ciprofloxacin (87.5%), piperacillin+tazobactum(83.4%). But all the AmpC producing strains were sensitive to imipenem [18].

Parveen R *et al.*, in their study observed 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 multi-drug resistance. Among the PMABL producers, (67%) had shown cefepime resistant. A total of 26 (10.7%) and13 (5.3%) isolates were resistant to meropenem and imipenem, respectively [20].

Antibiotic co-resistance was high in AmpC when compared to non-producers AmpC. This may be due to the fact that plasmids carrying these enzymes may carry co-resistance genes for other antibiotics. *Escherichia coli* are unique in that as it also expresses chromosomal Amp C at low levels [2]. The plasmid determined enzymes are very closely related to chromosomal Amp C β-lactamases, which confer resistance similar to their chromosomal Amp C β-lactamases.

Though three dimensional tests is the gold standard for AmpC detection, it is labour intensive and cannot be performed routinely on all clinical isolates. AmpC disc test can be used as a simple, convenient and rapid screening test for detection of AmpC β lactamase in clinical laboratories. Phenotypic tests are not able to differentiate between chromosomal *ampC* genes and *ampC* genes that are carried on plasmids or AmpC mediated resistance from other β-lactamase resistance mechanisms. A combination of phenotypic and molecular identification methods like Multiplex PCR is needed but the unavailability is limitation of our study.

CONCLUSION

In conclusion, the overall prevalence of 10.50% Amp C β-lactamase in *E. coli* and Multidrug resistance is a matter of concern. Dissemination of these organisms within the hospital or between the different regions of the country may become an important public health issue. So identification of AmpC may help in formulating the hospital infection control committee for guiding the physician to prescribe the most appropriate antibiotic, thus decreasing the selective pressure, which generates antibiotic resistance. Also continued surveillance of resistance among nosocomial pathogens and evolving, preventive measures aimed at reducing their spread.

CONFLICT OF INTERESTS

Declared None

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