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USE OF A MODIFIED DISC DIFFUSION TEST TO DETECT AMPC BETA LACTAMASE PRODUCTION IN *ESCHERICHIA COLI* FROM CASES OF URINARY TRACT INFECTIONS

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ABSTRACT

Routine detection of AmpC beta lactamases in *Escherichia coli* is cumbersome. This study was performed to determine the usefulness of a modified disc diffusion test to detect AmpC beta lactamases in *E.coli*. 28 non-repetitive, cefoxitin resistant isolates of *E.coli* were collected from cases of urinary tract infections and screened phenotypically for ESBLs and AmpC beta lactamase using DDST (ceftazidime and ceftazidime-clavulanic acid for detection of ESBLs, cefoxitin-cloxacillin DDST and cefoxitin -phenylboronic acid, cefoxitin-EDTA combination discs). Genotypic detection of AmpC and ESBL genes was done by PCR along with mutation analysis of chromosomal promoter of AmpC. Of the 28 isolates, 6 strains showed enhancement of zone of inhibition in cefoxitin-cloxacillin, cefoxitin plus 400mcg of phenyl boronic acid and a combination of 400 mcg of phenyl boronic acid and 292 mcg of EDTA but not with EDTA alone. Of the 6 phenotypically AmpC beta lactamase positive strains, 4 carried plasmid AmpC beta lactamase gene.

KEYWORDS: **Antibiotics, *Escherichia coli*, ESBL, AmpC beta lactamases, DDST**

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INTRODUCTION

Beta lactam antibiotics are one of the versatile groups of antibiotics invented by mankind and they still remain as one of the most common antibiotics used in clinical practice for treating variety of infections owing to their broad spectrum of activity, ease of delivery and minimal side effects. However, widespread and irrational use of antibiotics had led to the emergence of drug resistant bacteria. Resistance to a particular class of antibiotics may be inherited or acquired. Inherited resistance is not of major concern since those antibiotics to which the organism is resistant is not used routinely. On the contrary acquired resistance is of major concern as in the case of beta lactam antibiotics. There are four mechanisms, by which a bacterium develops resistance to beta lactam drugs- production of beta lactam hydrolyzing beta lactamase enzyme, alteration in the intracellular target of the drug i.e production of altered penicillin binding proteins, presence of efflux pumps and decreased expression of outer membrane protein.[1, 2] Beta lactamases are produced by several bacteria as a mode of self defense against various beta-lactam drugs like penicillins, cephalosporins and carbapenems. Extended spectrum beta lactamase (ESBL), Amp C beta lactamases and Carbapenemases are among the various clinically significant beta lactamases. Prevalence of multidrug resistant Gram negative bacilli is increasingly seen over the past few years and bacterial strains producing extended spectrum Amp C beta lactamases and extended spectrum beta lactamase are of particular concern. Extended spectrum beta lactamases are beta lactamases that confer resistance to penicillins, oxyimino cephalosporins (e.g., ceftriaxone, Ceftazidime, cefotaxime) and monobactams are inhibited by beta lactamase inhibitor combinations.[3] Amp C beta lactamases are clinically important cephalosporinases that confer resistanketsce to wide variety of beta lactam drugs and can be differentiated from ESBLs by their ability to hydrolyze cephamycins and are poorly inhibited by beta lactamase combinations[4]. In contrast to ESBLs, Cloxacillin and 3-aminophenyl boronic acid inhibit Ampc beta lactamases.[5-7] In Gram negative bacteria, these cephalosporinases can be either plasmid borne or chromosomally mediated. The plasmid borne AmpC beta lactamase was first reported in 1988 and are thought to be evolved through the movement of chromosomal genes found in various organisms like *Escherichia coli, Klebsiella pneumoniae, Enteobacter sp* etc. onto the plasmids.[3,8] Chromosomal AmpC is usually constitutively expressed at very low level. Genes for Amp C beta lactamase are found among the members of Enteobacteriaceae family viz. *Enterobacter, Shigella, Citrobacter freundii, Morganella morganii, Serratia marcescens* and *Escherichia coli* which can be induced by cefoxitin, cefotetan or imipenem with the expression being regulated by the regulator AmpR.[9] In the present study all the cefoxitin resistant strains of *E.coli* isolated from patients with urinary tract infections were analyzed for the presence of ESBL and AmpC beta lactamase. Simultaneously PCR was performed for all the strains for the presence of plasmid mediated Amp C beta lactamase genes and ESBLs and were characterized genetically by mutational analysis of AmpC promoter/attenuator gene for the presence of chromosomal AmpC. In the present study we wanted to evaluate the usefulness of a combination of antibiotic discs with enzyme inhibitors to detect both plasmid mediated as well as chromosomally mediated AmpC beta lactamases in *E. coli*.

MATERIALS AND METHODS

In this study, a total of 28 consecutive, non-repetitive, cefoxitin resistant urinary isolates of *Escherichia coli* were collected from cases of urinary tract infections in the Microbiology laboratory, JIPMER, Puducherry. These strains were collected over a time period of 15 months from September 2013 to November 2014. The isolates were identified by their colony characteristics and by conventional biochemical tests. Antibiotic susceptibility testing of the isolates was done by Kirby-Bauer disk diffusion method according to the criteria published by the Clinical Laboratories Standards Institute January 2014 guidelines for performance standards for antimicrobial susceptibility testing (CLSI). All antibiotic discs used were from Himedia (Himedia, Mumbai, India). Those isolates which showed decreased (intermediate/ resistant) susceptibility to cephamycins indicated by cefoxitin 30mcg (≤18mm) were included irrespective of their susceptibility to other antibiotics. Susceptibility testing was done on Mueller-Hinton agar using 0.5 Mc Farland standard inoculum densities. *Escherichia coli* ATCC 25922 was used as control in the antibiotic disc diffusion methods mentioned. Detection of the presence of ESBLs and AmpC beta lactamases were done both phenotypically and genotypically.[15] The phenotypic confirmation of ESBL was done by double disc synergy test (DDST) using ceftazidime 30mcg disks alone and in combination with clavulanic acid 10 mcg, with an increase in the inhibition zone diameter of ≥ 5mm around the combination disc compared to the disc containing ceftazidime alone was considered as positive. Currently there are no CLSI guidelines available for detection of AmpC beta lactamases. Phenotypic confirmation of AmpC beta-lactamase was performed using cefoxitin-cloxacillin double disc synergy test. The cefoxitin-cloxacillin double disc synergy test (CC-DDS) was performed using cefoxitin 30mcg and cefoxitin 30mcg plus cloxacillin 200mcg (Himedia, Mumbai, India). The strains were inoculated onto Mueller-Hinton agar using an inoculum of the test organism at McFarland 0.5 opacity and incubated at 35°C for 16 to 18 h. A difference of >4mm in the cefoxitin-cloxacillin inhibition zones compared to that of the cefoxitin alone zones was considered as indicative of AmpC production. Detection of Amp C beta lactamases was also done according to the Modified CLSI method for the detection of AmpC beta lactamases and ESBLs.[16] According to the modified CLSI method, phenotypic detection of AmpC was done using cefoxitin disc alone and with 400μg of phenyl boronic acid or 292μg of EDTA or both 400μg of phenyl boronic acid and 292μg of EDTA. The test was performed by inoculating onto Mueller Hinton agar of the test strain of the opacity of Mc Farland standard 0.5. Plates were incubated at 37°C for 18 hrs. An increase in the zone of inhibition of ≥5mm in the zone of inhibition of either cefoxitin containing boronic acid or EDTA or both

indicates the presence of AmpC beta lactamase production. DNA was extracted from colonies grown on MacConkey agar by boiling method. A multiplex PCR was performed for the detection of 6 families of plasmid mediated Amp C beta lactamase genes using the primers and the thermocycling conditions as per in Perez-perez.[17] A multiplex PCR was also performed for the detection of common ESBLS viz- *bla* CTXM, *bla*TEM & *bla* SHV.[18,19] AmpC promoter sequence analysis: PCR was performed using the following primers for detecting the promoter region mutation.[15] Once the amplicon of expected band size was obtained (271bp) the same was sent for sequencing to (Bioserve Biotechnologies, India). The primers used were AB1(5'-GATCGTTCTGCCGCTGTG-3') and ampC2 (5'-GGGCAGCAAATGTGGAGCAA-3'). The ampC promoter sequences were compared to the wild-type ampC sequence of *E. coli* strain ATCC 25922.

RESULTS

A total of 28 *Escherichia coli* isolates were collected from cases of urinary tract infections during the time period of 15 months based on the decreased susceptibility of the organism to cefoxitin (≤18mm) or the presence of indentation using cefoxitin as the inducing agent in the routine Kirby Bauer disc diffusion method i.e. the screen test, irrespective of the sensitivity to other antibiotics. These isolates were further characterized phenotypically and genotypically for the presence of AmpC beta lactamases and ESBLs. Antimicrobial susceptibility profile of all the 28 *Escherichia coli* isolates are summarized in table1 and it clearly shows that all the isolates were resistant to third generation cephalosporins and cefoperazone sulbactam but were sensitive to the other antibiotics routinely tested including aminoglycosides, quinolones, nitrofurantoin and carbapenems. Detection of the presence of ESBLs by both phenotypic and genotypic methods revealed that none of the strains harbored bla_{TEM}, bla_{SHV} *or* bla_{CTXM}ESBL genes.Of the 28 cefoxitin isolates, only 6 strains showed enhancement of zone of inhibition in cefoxitin plus 400mcg of phenyl boronic acid and a combination of cefoxitin with 400 mcg of phenyl boronic acid and 292 mcg of EDTA but not with the combination of cefoxitin with EDTA. The concentration of boronic acid and EDTA used in the study as mentioned earlier did not have any inhibitory effect on the bacterial growth. PCR was performed for all the 28 isolates for the detection of both plasmid mediated and chromosomal mediated AmpC beta lactamases irrespective of the presence or absence of ESBLs. Of the 6 which has shown decreased susceptibility to cefoxitin and enhancement of zone of inhibition of cefoxitin with phenyl boronic acid and combination of phenyl boronic acid and EDTA, only 4 isolates showed positivity results for Amp C beta lactamases by PCR. On sequencing these amplicons detected had 99% identity with *Escherichia coli* strain DHA-1 gene (Genome accession no: HQ188691.1.) The findings obtained by various phenotypic and genotypic tests for detection of AmpC are summarized in Table no.2.

Table1
Antimicrobial susceptibility profile of all the 28 Escherichia coli isolates

Number	Amikacin/Gentamicin/ Ciprofloxacin/Nitrofurantoin/Cefepime/Meropenem	Cefoperazone sulbactum	Ceftazidime	Ceftriaxone
No. sensitive	28	0	0	0
No. resistant	0	28	28	28

Table 2
The findings obtained by various phenotypic and genotypic tests for detection of AmpC in the isolates studied.

Strain No	Inhibition zone diameter Cefoxitin (30mcg)	Inhibition zone diameter Cefoxitin (30mcg)plus cloxacillin (200mcg)	Inhibition zone diameter Cefoxitin (30mcg)plus Phenyl boronic acid	Cx +PBA +EdTA	Cx +EDTA	PCR for plasmid AmpC	PCR for promoter region mutation
9	6	6	Enhanced	Enhanced	No enhancement	+ for DHAM	NEGATIVE
10	6	6	Enhanced	Enhanced	No enhancement	NEGATIVE	NEGATIVE
14	6	6	Enhanced	Enhanced	No enhancement	+ for DHAM	NEGATIVE
15	6	6	Enhanced	Enhanced	No enhancement	+ for DHAM	NEGATIVE
22	6	6	Enhanced	Enhanced	No enhancement	NEGATIVE	NEGATIVE
28	6	6	Enhanced	Enhanced	No enhancement	+ for DHAM	NEGATIVE

DISCUSSION

Although the breakpoints for extended cephalosporins for Enterobacteriaceae have been lowered, there remains a need for detecting the various resistance determinants such as ESBLs, AmpC beta lactamase and carbapenemase mainly for the epidemiological and infection control policies.[14,17,20] Despite there being several studies on AmpC beta lactamase production in *Escherichia coli*, the true incidence of such mechanism remains unknown. Moreover, testing for the presence of AmpC beta lactamase genes in all clinical isolates of

E.coli may not be practically feasible.[4,9,14,21,22] AmpC expression in *Escherichia coli* differs significantly from other organisms. In *E.coli*, the regulator AmpR is absent and Amp C is regulated by a weak promoter and strong attenuator resulting in low level constitutive expression of chromosomal AmpC. Hence the expression of AmpC is not inducible. Studies have found that Mutations in the AmpC promoter/attenuator region or over production of Amp C gene have been found to cause AmpC over expression in E.coli.[10-13] In addition to chromosomal AmpC, *E.coli* also contain plasmid AmpC which are transferred from the other *Enterobacteriacea*e family through horizontal gene transfer.[5,14] Different Phenotypic tests for the detection of AmpC beta lactamase have been described in the literature. But accurate phenotypic detection of AmpC is difficult since there are no standardized methods integrating the screening and confirmatory method currently available for detection.[15] Several AmpC beta lactamase detection tests are currently available. In this study, we have evaluated three phenotypic tests viz. decreased susceptibility to cefoxitin as the screening test, cefoxitin-cloxacillin double disc synergy test, and inhibitor assay based on phenyl boronic acid and EDTA. All the strains were further analyzed genotypically for the presence of ESBL, plasmid mediated AmpC and AmpC promoter region mutations for chromosomal AmpC overproduction. Several screening tests are available for the detection of AmpC beta lactamase. In our study we have chosen decreased susceptibility to cefoxitin rather than cefotetan as the screening test because Peter-Getzlaff et al have found that in the study conducted by them using a screening criterion as decreased susceptibility to cefoxitin (≤18mm), they could detect more than 90 % of the Amp C positive isolates. However, ACC family of AmpC beta lactamase would not be detected if cefoxitin is used as the marker in the screening test since they may appear cefoxitin susceptible.[6,14] In our study, all 28 E.coli isolates were cefoxitin resistant and further genotypic analysis for the presence of plasmid mediated AmpC beta lactamase could not detect any ACC family of AmpC beta lactamases. All the 6 isolates which were cefoxitin resistant have shown enhanced zone with cefoxitin – cloxacillin disc combination and also with phenyl boronic acid both of which are inhibitors of AmpC beta lactamases. This is in accordance with other studies which also has shown that cloxacillin and phenyl boronic acid are good substrates that can be used in the inhibitor based assays.[16,23,24] In the present study, of the six isolates which were cefoxitin resistant, produced enhanced zone with cefoxitin-cloxacillin double disc synergy test and inhibitor assay based on phenyl boronic acid, only four isolates revealed the presence of plasmid detected AmpC beta lactamase gene. Neither plasmid mediated AmpC genes nor hyper producing chromosomal Amp C genes could be detected in the remaining two isolates. Possibility of other AmpC beta lactamase gene family, outer membrane porin loss or presence of efflux pumps could be considered in this. There is paucity of data from the Indian laboratories on the prevalence of AmpC beta lactamase genes in *E.coli.* More extensive study focusing on outer membrane porin loss and efflux pumps need to be carried out to emphasize the importance of these mechanisms of resistance contributing to multi drug resistance in *E.coli* isolates. Extended spectrum AmpC beta lactamases hydrolyze even fourth generation cephalosporin, they can be only inhibited by carbapenems. This is mediated by certain mutations in the promoter or the attenuator regions in the chromosome.[25] In this study, all the isolates were sensitive to cefepime and we could not detect any mutations in these regions. This would add to the selection pressure on carbapenem resistance. They are often resistant to multiple classes of antibiotics making the right choice of antibiotic for therapy difficult. There is still no effective inhibitor which can be used safely in face of an infection with an AmpC beta lactamase producing strain, though tazobactum combinations have been tried but with limited success.[5]

CONCLUSION

Prevalence of multi drug resistant organisms is increasing worldwide. Infections caused by these isolates are associated with adverse outcomes in terms of morbidity and mortality and increase in duration of hospital stay mainly because of the lack of drugs to treat these infections. It is necessary to routinely test all the organisms for the presence of ESBLs and AmpC beta lactamase genes since the exact prevalence of the resistant organisms in a hospital setting is essential for formulating the infection control policies. This would help in the judicial use of antibiotics for the treatment and thereby preventing the development of `Superbugs`.

LIMITATIONS OF THE STUDY

Our study has several limitations. We have selected only those *E. coli* isolates causing urinary tract infections which were cefoxitin resistant. So the exact prevalence of AmpC beta lactamase and ESBL producing *E.coli* isolates in our hospital setting could not be calculated. Secondly the geographical distribution of plasmid encoded AmpC beta lactamase genes vary widely and the performance of the phenotypic tests also varies significantly among different families of prokaryotes. The other mechanisms of resistance like outer membrane porin loss and presence of efflux pumps were not looked for.Finally it is possible that the other AmpC beta lactamases may not have been detected by the Multiplex PCR that we used in this study.

CONFLICT OF INTEREST

Conflict of interest declared none.

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