Detection of AmpC β-Lactamases among Gram Negative Clinical Isolates

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Research Article

Abstract: **Background and Objectives:** AmpC β-lactamases confer resistance to a wide variety of β-lactam antibiotics including cephamycins such as cefoxitin and cefotetan, narrow and broad spectrum cephalosporins, aztreonam and are poorly inhibited by β-lactamase inhibitors such as clavulanic acid. The present study was undertaken to determine the occurrence of AmpC enzyme producing Gram negative clinical isolates at Navodaya Medical College, Raichur. **Materials and Methods:** 200 non-repeat Gram negative clinical isolates from various samples were screened for AmpC β- lactamases by using Modified disc Approximation method. The "Screen positive" isolates were detected for AmpC β-lactamase by Modified three-dimensional enzyme extract test, AmpC disk test, and phenyl boronic acid test. **Results:** Out of the 200 Gram negative isolates, 87 (43.5%) isolates were screened to be positive and 10 (5%) isolates were inducible AmpC producers. Of these 97 isolates subjected to confirmatory tests, 74 (37%) were harbouring AmpC enzymes. Majority of AmpC positive isolates were *E.coli* (20.5%), *K.pneumoniae* (5.5%) and *Acinetobacter* spp. (3.5%). **Conclusion:** Phenyl boronic acid test is the simple and practical method for the detection of AmpC in routine clinical microbiology laboratories. Plasmid-mediated AmpC β-lactamases have been associated with false *in vitro* susceptibility to cephalosporins. Thus, the type of β-lactamase produced by the organism should be detected along with the antibiogram for appropriate treatment of the patient.

Keywords: AmpC β-lactamase, cefoxitin, Gram negative bacteria.

Introduction

A common mechanism of bacterial resistance to beta lactam antibiotics is the production of β-lactamase enzymes that cleave the structural β-lactam ring of these drugs[1]. Over the last two decades many new β-lactams have been developed that were specifically designed to be resistant to hydrolytic actions of β-lactamase[2]. A new type of β-lactamase has emerged, even to this class of drugs. AmpC β-lactamase is one of the new types of β-lactamases[2]. AmpC β-lactamases are cephalosporinases, which belong to the molecular class C as classified by Ambler in 1980[3] and Group1 under a classification scheme of Bush *et al.,*[4]. These are clinically significant as they may confer resistance to a wide variety of β-lactam drugs, including α-methoxy-β-lactams like cefoxitin, cefotetan; narrow, expanded and broad spectrum cephalosporins; aztreonam, a monobactum and most significantly β-lactam plus β-lactamase inhibitor combinations (viz, amoxycillin-clavulanic acid, piperacillin-tazobactum, etc) [3]. Although it has been over a decade since Plasmid-mediated AmpC β-lactamases were discovered, most clinical laboratories and physicians remain unaware of their clinical importance[5]. As a result, organisms producing these types of β-lactamases often go undetected and therefore have been responsible for several nosocomial outbreaks. Detecting AmpC isolates is clinically important, not just because of their broader cephalosporin resistance, but also because carbapenem resistance can arise in such strains by further mutations, resulting in reduced porin expression[6]. There are no recommended Clinical and Laboratory Standards Institute (CLSI) guidelines for the detection ofAmpC β-lactamases and clinical laboratories need to address this issue as much as the detection of Extended-spectrum β-lactamases (ESBLs), since both may co-exist and mask the detection of ESBL[7].Without accurate laboratory detection and reporting of such resistant phenotypes and strains producing Plasmid-mediated AmpC β-lactamases, treatment of Gram negative infection may remain suboptimal. The present study was undertaken to determine the occurrence of AmpC enzyme producing Gram negative clinical isolates and to evaluate the efficacy of three dimensional extract test, AmpC disk test and phenyl boronic acid test in detecting AmpC. The antibiotic sensitivity pattern of Gram negative isolates was determined to enhance infection control and to ensure effective therapeutic options.

Materials and Methods

The present study was carried out in the Department of Microbiology, Navodaya Medical College, Raichur from November 2010–October 2011. Ethical clearance was obtained from the Institutional Ethical Committee, before starting the study. A total of 200 non-

repeat Gram negative clinical isolates from various clinical samples like urine, sputum, pus and others constituted the study material. The isolates were identified by biochemical tests. The antibiotic sensitivity test was performed according to CLSI guidelines[8]

Screening for AmpC by Modified double disc approximation method

In order to screen AmpC, a modified double disk approximation method (MDDM), was done. A 0.5 McFarland of Gram negative isolate was swabbed on Mueller Hinton Agar (MHA) plates and disk of cefotaxime (30µg) and ceftazidime (30µg) were placed adjacent to Cefoxitin (30µg) disk at a distance of 20mm from each other. After incubation, isolates showing blunting of ceftazidime or cefotaxime zone of inhibition adjacent to cefoxitin disk (Inducible AmpC) [Fig-1] or showing reduced susceptibility to either of the above test drugs (ceftazidime or cefotaxime) and cefoxitin[<18mm] were considered as "screen positive" and selected for detection of AmpC β-lactamases. Simultaneous detection of ESBL was done by using ceftazidime/clavulanic acid (30/10µg). After incubation, an enhanced zone of inhibition[≥5mm] around ceftazidime/clavulanic acid in comparison with a disc with ceftazidime disc alone was confirmed as ESBL.*K. pneumoniaeATCC 700603* and *Escherichia coli ATCC 25922* were used as positive and negative controls, respectively.[9]

DetectionofAmpC β-lactamases

Modified three-dimensional extract test

The "screen positive" isolates were subjected for the detection of AmpC enzyme by modified three-dimensional extract test. Briefly, fresh overnight growth from MHA plate was transferred to a pre-weighed sterile microcentrifuge tube. The tube was weighed again to determine the weight of bacterial mass to obtain 10–15mg of bacterial wet weight. The bacterial mass was suspended in peptone water and pelleted by centrifugation at 3000 rpm for 15 minutes. Crude enzyme extract was prepared by repeated freeze-thawing of the bacterial pellet (approximately 10 cycles). Lawn culture of *E. coli ATCC 25922* was prepared on MHA plates and cefoxitin (30µg) disks were placed on the plates. Linear slits (3cm) were cut using sterile surgical blade, 3mm away from cefoxitin disk. At the other end of the slit a small circular well was made and the enzyme extract was loaded. A total of 30 to 40µl of extract was loaded in the well at a 10-µl increment. The plates were kept upright for 5–10 minutestill the liquid dried and were incubate at 37°C for 24h. Isolates showing clear distortion of the zone of inhibition of cefoxitin were taken as AmpC producers.[9] [Fig-2]

AmpC disk test

All isolates subjected to three-dimensional test were also simultaneously checked by AmpC disk test.A lawn culture of *E. coli ATCC 25922* was prepared on MHA plate. Sterile disks (6mm) were moistened with sterile saline (20µl) and inoculated with several colonies of test organism. The inoculated disk was then placed beside a cefoxitin disk on the inoculated plate. The plates were incubated overnight at 35°C. A positive test appeared as a flattening or indentation of the cefoxitin inhibition zone in the vicinity of the test disk. A negative test had an undistorted zone.[9] [Fig-3]

Phenyl boronic acid (PBA) disc enhancement method

In this method,two cefoxitin discs (30µg) were placed on a MHA plate lawn inoculated with a 0.5 McFarland turbidity adjusted suspension of the test strain. To one of these discs, 400µg of phenyl boronic acid (Sigma-Aldrich) was added. After overnight incubation at 37° C, the zones of inhibition were measured. Enhancement of zone of inhibition by 5mm around a cefoxitin disc with PBA, in comparison with a disc with cefoxitin alone, was taken as a positive result for AmpC production.[10] [Fig-4]

Results

Statistical Analysis: Descriptive statistics such as proportions and graph are used to present the data. Differences between proportions were analysed using $\chi 2$ tests, or by Fisher's exact test if sample sizes were small or unbalanced. A two tailed P-value < 0.05 is considered statistically significant. Out of 200 Gram negative clinical isolates, 196 isolates were sensitive to imipenem (98%), followed by 158 isolates to netilmycin (79%), 139 to amikacin (69.5%), 105 to cefoxitin (52.5%), and 99 to gentamycin (49.5%). Cefoperazone/sulbactum and piperacillin/tazobactum showed a sensitivity of 32% (64 isolates). Total of 187 isolates were resistant to amoxyclav (93.5%), followed by cotrimoxazole (182 isolates,76%), cefepime (141 isolates, 70.5%), ceftazidime (131 isolates, 65.5%), aztreonam (120 isolates, 60%), ciprofloxacin (112 isolates, 56%) and cefotaxime (86 isolates, 43%). Table-1 shows the results of screening test. AmpC were commonly seen in *E.coli*, 41(20.5%); followed by *Klebsiella pneumoniae*(5.5%), *Acinetobacter* sp. (3.5%), *Pseudomonas aeruginosa* (3%), *Citrobacter* spp(1.5%), *K.oxytoca* (1%), Atypical *E.coli*, *Proteus mirabilis*, *P.vulgaris* and *Serratia marcescens*(0.5% each). Co-existence of ESBL and AmpC was seen in 38 isolates (19%).

Discussion

Constitutive overexpression of AmpC β-lactamases in gram negative organisms occurs either by de-regulation of the ampC chromosomal gene or by acquisition of a transferable ampC gene on a plasmid or other transferable

element. The transferable ampC gene products are commonly called plasmid-mediated AmpC β-lactamases. Organisms that constitutively overexpress the chromosomal genes are collectively called 'de-repressed mutants'[11]. The present study showed 43.5% isolates to be resistant to cefoxitin and taken as screening positive and 5% were inducible AmpC subjected to confirmation as AmpC producer. But the results of Manchanda *et al.,*[12] showed that screening should include all the clinical isolates showing resistance to any of the cephalosporins and/or aztreonam, irrespective of their cefoxitin susceptibility status. Although screening methods that use cefoxitin to detect AmpC harbouring isolates are useful, but not sensitive[12]. Our study showed 5% of the isolates as inducible AmpC producers, in *Pseudomonas aeruginosa* (2%), *Klebsiella* spp. (1%), *E.coli* (1%), *Acinetobacter* sp. (0.5%) and *Serratia marcescens* (0.5%). Inducible AmpC is rare in *E.coli* and *Acinetobacter* spp. [13] but it is reported in our study. Plasmid mediated inducible AmpC found in our study, is reported to be extremely rare in *Klebsiella* spp[13]. In this study, out of the 97 screen positive isolates, 74 (76.29%) isolates were confirmed as AmpC producers. The cefoxitin resistance in remaining 23 (23.71%) isolates could be due to some other resistance mechanism, such as lack of permeation of porins[13]. The prevalence of AmpC β-lactamase in our study was lower than other studies[13,14]. This could be due to infrequent use of higher antibiotics in our hospital resulting in reduced selection pressure. Our findings co-related with that of Rudresh *et al.*[15]. The de-repressed mutants far exceeded the inducible AmpC producers in this study. In the present study, AmpC prevalence is more in IPD patients (40.44%) compared to OPD cases (29.69%). AmpC producing isolates have been commonly encountered in patients after prolonged hospitalization in ICU, following surgical procedures or those who had an underlying disease or who were immunocompromised[14]. Table-2 shows the age-wise distribution of AmpC β-lactamases. AmpC was more commonly seen in age group ≥70 years (50%) and ≤10 years (42.86%). Higher rate of AmpC prevalence in age group ≤10 years may lead to high susceptibility to infection along with their weak immune system. In higher age group (≥70 yrs) AmpC existence can cause high rate of infection along with debilitating condition and weakened immunity[13]. In our study, 43 (21.5%) isolates were positive for AmpC by (three-dimension enzyme test) 3DET. In AmpC disk test 40 (20%) isolates were positive for AmpC. The AmpC disk test detected 3 isolates which were missed by 3DET. The 3DET detected 6 more isolates compared toAmpC disk test. Total of 74 (37%) isolates were found as AmpC producers by PBA test. Total of 5 isolates positive by 3DET were negative by PBA test. The positivity in 3DET can be due to porin channel loss13. So PBA test appears more specific compared to 3DET. Also it detected 36 (18%) more isolates compared to 3DET. The earlier studies[16,17,18,19] reported that inhibitor based tests are very simple, highly sensitive and specific for the identification of bacteria producing class C β-lactamases. Our study also showed similar findings which is statistically significant (P<0.05). This test is a simple, practical and efficient method that uses current CLSI methodology to detect plasmid-mediated AmpC β-lactamase in organisms that usually donot harbor genes for these enzymes. Therefore, isolates that are positive by PBA test should be reported as being resistant to all cephalosporins[14].

Conclusion

The present study showed a high prevalence (37%) of AmpC β-lactamases in Gram negative bacteria. Among the three methods employed to confirm AmpC β-lactamases, PBA test was more sensitive and specific. The AmpC disk test is relatively easier to perform and gives similar results, when compared to modified 3DET and can be used in routine microbiology laboratories.

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Table 1: AMPC screening positive isolates and inducible AMPC among total isolates

Screening test	Frequency	Percent
Negative	103	51.5%
Positive	87	43.5%
Inducible	10	5.0%
Total	**200**	**100%**

The Table-1 shows that among 200 Gram negative isolates, 87 (43.5%) were screening positive, 10 (5%) were inducible AmpC and rest 103 (51.5%) were screening negative.

Table 2: Age wise distribution of AMPC B-Lactamases

Age (Yrs)	Negative	Positive	Total
≤ 10	12	9(42.86%)	21
11-20	12	7(36.84%)	19
21-30	25	13(34.21%)	38
31-40	17	11(39.28%)	28
41-50	23	13(36.11%)	36
51-60	18	10(35.72%)	28
61-70	15	7(31.82%)	22
≥ 70	4	4(50%)	8
Total	**126**	**74(37%)**	**200**

The Table-2, shows that AmpC was more commonly seen in age group ≥70 years (50%) and ≤10 years (42.86%). Followed by age groups 31-40 (39.28%), 11-20 (36.84%), 41-50 (36.11%), 51-60 (35.72%), 21-30 (34.21%) and 61-70 (31.82%).

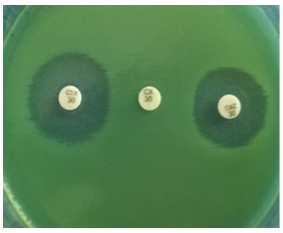

Figure 1: Inducible Amp C Beta Lactamase

Figure 2: Modified Three Dimension Enzyme

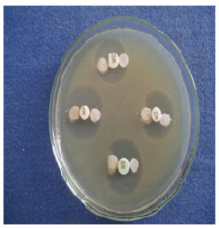

Figure 3: Amp C Disk Test

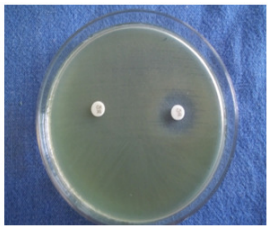

Figure 4: Phenyl Boronic Acid Test