Detection of Amp C beta lactamases production in clinical isolates of Escherichia coli and Klebsiella pneumoniae using Inhibitor based method

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Abstract: Amp C enzymes can be delineated from extended spectrum beta lactamases by their ability to hydrolyse cephamycins as well as extended spectrum cephalosporins. They are reported with increasing frequency, but the rate of its occurrence remains unclear. This study was undertaken to determine the occurrence of AmpC enzymes harbouring Escherichia coli and Klebsiella pneumoniae clinical isolates from our hospital. A total of 150 clinical isolates were subjected to susceptibility test with cefoxitin and third generation cephalosporin (3GC) antibiotics ampicillin, amikacin, cotonaxazole, gentamycin, meropenem, imipenem, ertapenem and tetracycline by disc diffusion method. Those isolates which were resistant to 3GC and cefoxitin were tested with Amp c disc method, Modified three dimension test, Inhibitor based method using Boronic acid for confirming AmpC beta lactamase. Among the 150 isolates 9 (6) isolates were cefoxitin resistant indicating AmpC production. Plasmid mediated AmpC was detected in 8 (88.8) of the 9 isolate which were cefoxitin resistant by Inhibitor base method using boronic acid. AmpC producers (77.7) were also ESBL enzyme producers. AmpC production was observed in total 10.66 E.coli and 1.3 K.pneumoniae. The Inhibitor based method using boronic acid is useful in identifying AmpC producers. Among the 150 isolates 99 (66.6) of them were Extended spectrum beta lactamase producers.

Keyword: Amp C beta lactamases, ESBL, Inhibitor based test using boronic acid

Introduction: Antibiotics resistance is one of the main problems faced by physicians in treating an infectious disease. Amp C beta lactamases is one of the mediators of antibacterial resistance in Gram negative bacilli. They are cephalosporinas, which are not inhibited by clavulenic acid, hydrolize cephamycins and other extended spectrum beta lactams.

Amp C beta lactamases are either Chromosomal (inducible Amp C) or plasmid mediated. Chromosomally mediated Amp C have been demonstrated in Acinetobacter spp., Aeromonas spp., Chromobacterium violaceum, Citrobacter freundii, Enterobacter spp., E.coli, Hafnia alvei, Morganella morganii, Providentia rettgeri, Yersinia enterocolitica. Numerous pathogens produce plasmid encoded Amp c beta lactamases e.g. Escherichia coli, Klebsiella pneumonia, Salmonella spp., Proteus mirabilis and Citrobacter freundii.

True incidence of Amp C lactam remains unknown, particularly in India baring few studies. Chromosomal Amp C is inducible in most genera of Enterobacteriaceae family. Plasmid mediated AmpC unlike chromosomal mediated are mostly expressed constitutively. Cefoxitin disc diffusion with zone diameter < 18mm is used as screen test for AmpC detection. Those isolates which are cefoxitin resistant were further confirmed for AmpC by using AmpC disc test. Modified three dimension test, Inhibitor based test using boronic acid. Methods for detecting AmpC lactamases in clinical laboratory still are evolving. This is due to lack of standard guidelines for its detection. Patients affected with Amp C lactamase producers have a prolonged hospital stay, and increased morbidity and mortality. Therefore detection of Amp C lactamase is very important in the management of such patients. A prospective study was undertaken to determine the occurrence of Amp C lactamases in Escherichia coli and Klebsiella pneumoniae in our Hospital.

Materials and method: This study included 150 non repetitive clinical isolates of Escherichia coli (n-75) and Klebsiella pneumoniae (n-75) which was collected over a period of 3 months (October to December 2011) from our hospital. These isolates were obtained from both inpatients and outpatients. Isolates were identified by standard protocols. The antibiogram of the isolates were determined by the standard Kirby Bauer’s disc diffusion method. Antibacterial susceptibility testing were carried out by the disc diffusion method as per CLSI guidelines. The antibiotics tested were: Ampicillin (10 µg), Cefotaxime (30 µg), Ceftriaxone (30 µg), Cephaloridine (30 µg), Cefepime (30 µg), Ceftriaxone (30 µg), Gentamicin (5 µg), Amikacin (30 µg), Piperacillin (100 µg), piperacillin + tazobactam (10 µg), Cefoxitin (30 µg), Imipenem (10 µg), Meropenem (10 µg), Ertapenem (10 µg).

ESBL and Amp C: All isolates were tested for ESBL production by Cefazidime and Cefotaxime (30µg) disc with and without clavulanic acid (10µg). Zone size 5 mm for either antimicrobial agent tested with and without clavulanic acid were taken as ESBL producers. And Cefoxitin resistant strains < 18 mm were taken as AmpC lactamase producers.
Modified three dimension test

Fresh overnight growth from Muller Hinton agar was emulsified in a test tube containing peptone water. Pellets obtained after centrifugation at 3000 rpm for 15 minutes were subjected 7 times to freezing and thawing to obtain crude enzyme extract. Lawn culture was made by Escherichia coli ATCC 25922 on Muller Hinton agar plate. With a sterile blade a linear slit of 3cm were cut about 2-3 mm away from the Cefoxitin disc. The outer edge of the slit was stabbed with sterile pipette and crude enzyme extract was filled into it till top. The plate was kept upright for 10 – 15 minutes until the solution dried and then was incubated at 37°C overnight. The isolates showing distortion of zone of inhibition of cefoxitin were taken as Amp C producers.

Amp C disc test

A disc containing cefoxitin 30g was placed on a lawn culture of E.coli ATCC25922 in Muller Hinton agar medium. A sterile disc moistened with sterile saline (20l) inoculated with several colonies of test organisms was placed beside the cefoxitin disc followed by overnight incubation at 37°C. Indentation of cefoxitin zone in vicinity of test strain disc was taken as positive test.

Inhibitor based method using Boronic acid

A disk containing 30 g of cefoxitin and another containing 30 g of cefoxitin with 400 g of boronic acid were placed on lawn culture of test organism in Muller Hinton agar and incubated overnight at 37°C. Difference of 5 mm of zone diameter between the two discs were taken as positive for Amp C production.

Result:

Out of 150 isolates tested 127 (84.6%) were from inpatients and 23(15.3%) were from outpatients. Fifty-one of them (34%) were susceptible mostly to all antibiotics. Resistance to third generation cephalosporins was observed in 99(66%) isolates, of which 9 (6%) of them were cefoxitin resistant indicating presence of Amp C lactamase. All the 99 isolates were ESBL producers by disc potentiation test. 7 out of 9 isolates which showed cefoxitin resistance also exhibited zone of enhancement with clavulanic acid, indicating both ESBL and Amp C producers by disc potentiation test. 7 out of 9 isolates which were cefoxitin resistant indicating both ESBL and Amp C producers by disc potentiation test. 7 out of 9 isolates which were cefoxitin resistant indicating both ESBL and Amp C producers by disc potentiation test. 7 out of 9 isolates which were cefoxitin resistant indicating both ESBL and Amp C producers by disc potentiation test. 7 out of 9 isolates which were cefoxitin resistant indicating both ESBL and Amp C producers by disc potentiation test. 7 out of 9 isolates which were cefoxitin resistant indicating both ESBL and Amp C producers by disc potentiation test.

Table 1: Distribution of Amp C and ESBL in test isolates

<table>
<thead>
<tr>
<th>Mechanism of resistance</th>
<th>E.coli (75)</th>
<th>K.pneumoniae (51)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESBL</td>
<td>51 (68%)</td>
<td>48 (64%)</td>
</tr>
<tr>
<td>Non ESBL</td>
<td>24(32%)</td>
<td>27 (33%)</td>
</tr>
<tr>
<td>ESBL + Amp C</td>
<td>6 (8%)</td>
<td>11 (13%)</td>
</tr>
<tr>
<td>Amp C</td>
<td>2 (2%)</td>
<td>Nl</td>
</tr>
</tbody>
</table>

Cefoxitin screened positive AmpC lactamase, 9 isolates where tested with three other methods. Inhibitor based test with boronic acid identified 8 (88.8%), Modified three dimension test 7 (77.7%), Amp c disc test – 6(66%) isolates (Table-2)

Table 2: Results of different methods of Amp C testing

<table>
<thead>
<tr>
<th>Test Name</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibitor based test (boronic acid)</td>
<td>8 (88.8%)</td>
</tr>
<tr>
<td>Modified three dimension test</td>
<td>1 (11.1%)</td>
</tr>
<tr>
<td>Amp C disc test</td>
<td>6 (66%)</td>
</tr>
</tbody>
</table>

Discussion:

AmpC beta lactamases are cephalosporinase belonging to molecular class C as classified by Ambler and to group I under classification scheme of Bush et al. They confer resistance to wide variety of lactam drugs including alpha methoxy lactam, cefoxitin, narrow, expanded and broad spectrum cephalosporins and aztreonam. The chromosomal AmpC gene were detected on plasmids and were transferred to organism which normally do not express chromosome lactams . Eg: Escherichia spp., Klebsiella spp., Salmonella spp. Plasmid mediated AmpC lactamases resistance mechanism has been found all over the world causing nosocomial outbreaks. Differentiation between these types of organism is a must as it will prevent the unnecessary usage of cephalosporins and carbapenems which results in the selective pressure driving the Amp C or plasmid mediated class A carbapenem resistance gene propogation. Currently, CLSI guidelines do not indicate the screening and confirmatory tests for detection of these beta lactamases. However cefoxitin resistance is used to screen the AmpC producers. There are several studies describing various test methods for Amp C detection like the three dimensional test, Amp c disc test, Modified double disc test, cefoxitin agar method, inhibitor based method using boronic acids. But none of them are considered as gold standard and so the phenotypic test are unreliable. Isoelectric focusing and genotyping characterization are considered as gold standard tests.

In this study, we screened E.coli and K.pneumoniae, the most common isolates in our laboratory. ESBL-producing isolates of E. coli and K. pneumoniae were obtained from inpatient units as well as outpatient clinics. ESBL producing E. coli was found in 47.8% of outpatients and 66.6% of inpatients where as ESBL producing K.pneumoniae was found in 4.3% of outpatients and 65.3% of inpatients. All the AmpC, harbouring organisms were found in clinical specimens from admitted patients. This shows that AmpC harbouring isolates are largely restricted to hospitalized patients only. AmpC beta lactamase were detected in 6 percent isolates, 77% of which occurred in combination with ESBLs. Hemalatha et al reported much higher prevalence (47.3%) of AmpC lactamases compare to our study. Singhal et al reported similar incidence (8%). Lower prevalence of 2.3% was observed in studies from West. There are different studies done on AmpC detection. Singhal et al suggests Amp C disk test as a more reliable and rapid method for detection of Amp C isolates. While another Indian study observed Modified three dimension test as more sensitive. Our result indicates that the inhibitor based method using boronic acid is more promising for amp C detection as this test has increased sensitivity compared to other test procedures but requires further standardization.

References:


