Detection and Characterization of AmpC B-Lactamases in Indian Clinical Isolates of *Escherichia coli*, *Klebsiella pneumonieae* and *Klebsiella oxytoaca*

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Abstract The aim of the study was to determine the prevalence of AmpC producing *E. coli*, *Klebsiella pneumoniae* and *Klebsiella oxytoaca* and to evaluate and compare four different phenotypic methods viz; Modified Three Dimensional Test, AmpC Disk Test, AmpC Disk Test with Tris-EDTA and Inhibitor based detection test for the detection of AmpCβ-lactamas in these isolates, collected during a period of 2003 to 2009 from various hospitals of Delhi, India. Characterization of these enzymes was carried out by polymerase chain reaction (PCR) as well as by nucleotide sequencing. Phenotypic results showed that Inhibitor based detection test, Modified three dimensional test and AmpC disk test detected 40.8% of *E. coli* and 34% of *Klebsiella* spp. to be AmpC producers. However, Inhibitor based detection test was found to be simple, highly specific and sensitive in detecting these enzyme producers. PCR and nucleotide sequencing showed the presence of blaCMY-2 and blaACT-1 genes in all the positive isolates.

Keywords AmpC β-lactamases, Boronic acid, CMY-2, ACT-1

1. Introduction

In the past few decades there has been a rise in the incidence of resistance to β-lactam antibiotics in bacterial pathogens. Gram-negative bacilli resistant to β-lactams are increasingly being isolated from seriously ill and hospitalized patients (1).

Of the various resistance mechanisms prevailing among bacteria, production of β-lactamases is the most widespread and effective mechanism through which bacteria can become resistant to β-lactam drugs.

With the increasing use of β-lactam drugs and introduction of various inhibitor combinations such as amoxicillin-clavulanic acid or sulbactam, Ambler class C and Bush group I β-lactamase enzyme, known as AmpC β-lactamases have emerged and are being reported worldwide with varying prevalence rates (2), (3) and (4). These enzymes confer resistance to penicillins, first, third generation cephalosporins, cephams and monobactams such as aztreonam. These enzymes are poorly inhibited by the commercially available β-lactamase inhibitors such as clavulanic acid, sulbactam etc. but are inhibited by cloxacillin and phenylboronic acid (5) and (6).

These enzymes can either be chromosomally mediated or encoded by plasmids. Plasmid mediated AmpC enzymes are expressed at high levels hence, contribute significantly to clinical β-lactam resistance. Moreover, clinical isolates possessing plasmid encoding AmpC enzymes often are resistant to multiple antibiotics hence, leaving few therapeutic options (5) and (7). Though, this enzyme has been reported in several bacteria, it is most prevalent in clinical isolates of *Escherichia coli* and *Klebsiella* spp. (8).

Several tests for detection of AmpC β-lactamases in gram-negative bacilli have been developed and employed by workers in different parts of the world with varying degrees of success (9) and (10). This includes various phenotypic tests as well some PCRs, however, no specific Clinical laboratory standards institute (CLSI) recommended method is available.

In many laboratories PCRs are still expensive tools to be used, hence looking for specific and sensitive phenotypic tests have always been a challenge. Even though PCR is a gold standard method to detect AmpC beta lactamase genes many clinical laboratories show interests in performing phenotypic tests as these are cost effective. Therefore, it is important to come up with the most sensitive and specific phenotypic test for detecting AmpC beta lactamase producing isolates.

In view of the paucity of studies comparing different phenotypic tests for the detection of AmpCβ-lactamas in...
clinical isolates of *Escherichia coli* and *Klebsiella* spp., the present study was undertaken to evaluate and compare various phenotypic methods in terms of their sensitivity and specificity for the detection of these enzymes in clinical isolates and to characterize these enzymes produced by these isolates by polymerase chain reaction (PCR) as well as by nucleotide sequencing of ampC genes.

### 2. Materials and Methods

#### 2.1. Bacterial Isolates

A total of 500 non-repetitive isolates; 250 each of *Klebsiella* spp. (*Klebsiella pneumoniae* = 199, *Klebsiella oxytoca* = 51) and *Escherichia coli* recovered from a variety of clinical specimens viz. pus, urine, sputum, wound swab, blood, tracheal aspirate, from various hospitals of Delhi, India were collected during a period of 2003 to 2009. All the isolates were identified by standard biochemical methods.

#### 2.2. Screening for AmpC Production

The isolates were screened for presumptive AmpC production by testing their susceptibility to cefoxitin (30 µg) using Kirby Bauer disk diffusion method (11). The inhibition zone sizes were interpreted as per the CLSI (formerly NCCLS) guidelines (12). All the isolates with an inhibition zone diameter of less than 18 mm were labeled as screen positive. These screen positive isolates were used for comparing different phenotypic methods.

#### 2.3. Phenotypic Tests for Detection of AmpC β-lactamases

All the screen positive isolates were subjected to four different phenotypic tests viz. Modified Three Dimensional Test (M3DT) (13), AmpC disk test (9), AmpC disk test with Tris-EDTA (14) and Inhibitor (Boronic acid) based detection test (10), for the detection of AmpC β-lactamases in these isolates.

*E. coli* C600 pMG230 was used as a positive control and *E. coli* ATCC 25922 as a negative control with each batch of tests.

##### 2.3.1. Modified three dimensional test (M3DT)

An overnight growth on Mueller Hinton Agar (MHA) plate was transferred to a preweighed sterile micro centrifuge tube to obtain 10-15 mg of bacterial wet weight. The bacterial mass was suspended in peptone water and pelleted by centrifugation at 3000 rpm at 4°C for 15 minutes. Crude enzyme extract was prepared by repeated freezing and thawing of the bacterial pellet. Lawn culture of *E. coli* ATCC 25922 was prepared on MHA plates and cefoxitin disc of 25922. A 30µg cefoxitin disc was placed on the inoculated MHA plate. A sterile disc of 6 mm moistened with 20µl of sterile saline was kept and several colonies of test organism were inoculated on this disc. A cefoxitin disc was placed next to this disc (almost touching) on the inoculated plate. The plates were incubated overnight at 37°C. A flattening or indentation of the cefoxitin inhibition zone in the vicinity of the disc was considered a positive test.

##### 2.3.2. AmpC Disc Test

A lawn culture of *E.coli* ATCC 25922 was prepared on MHA plate. A sterile disc of 6 mm moistened with 20µl of sterile saline was kept and several colonies of test organism were inoculated on this disc. A cefoxitin disc was placed next to this disc (almost touching) on the inoculated plate. The plates were incubated overnight at 37°C. A flattening or indentation of the cefoxitin inhibition zone in the vicinity of the disc was considered a positive test.

##### 2.3.3. AmpC disc test with Tris-EDTA

The AmpC discs were prepared by applying an aliquot of 20µl of a 1:1 mixture of normal saline and 100x Tris- EDTA to each of sterile filter paper discs, which were allowed to dry. The discs were stored at 2 to 8°C. The surface of a MHA plate was inoculated with a lawn culture of *E.coli* ATCC 25922. A 30µg cefoxitin disc was placed on the inoculated MHA plate. An AmpC disc was rehydrated with 20µl of saline and growth of several colonies of the test organism was applied on the disc using a sterile inoculating loop. The inoculated AmpC disc was placed with its inoculated surface in touch with the agar surface and in close proximity (almost touching) to the cefoxitin disc. The plate was incubated overnight at 37°C. An indentation or a flattening in the zone of inhibition of cefoxitin disc indicated a positive result.

##### 2.3.4. Inhibitor (boronic acid) based detection method

Screened positive isolates were also checked by this method for the presence of AmpC β-lactamases. Boronic acid was prepared by dissolving 120mg of phenyl boronic acid in 3ml DMSO, to which 3ml of sterile distilled water was added. Discs were prepared by dispensing 20µl of the stock solution on discs containing 30 µg of cefotetan. This test was performed by inoculating MHA plate by the standard disc diffusion method (CLSI/NCCLS), by placing a disc containing 30µg of cefotetan along with a disc containing 30µg of cefotetan and 400µg of boronic acid. The inoculated plates were incubated overnight at 35°C. Organism showing an increase of 5mm, of zone diameter around the disc containing cefotetan and boronic acid as compared to that of zone diameter around the disc of cefotetan alone was considered as AmpC producer.

#### 2.4. Detection and Characterization of AmpC β-lactamases

##### 2.4.1. Polymerase Chain Reaction (PCR)

Detection of plasmid mediated AmpC β-lactamase(ampC)
genes in all the screen positive isolates was carried out by PCR as described by Perez and Hanson, 2002 (15). Briefly, PCR reactions were performed in a final volume of 50 µl of the amplification mixture containing 1.25 U of Taq DNA polymerase, 1X Taq buffer, 0.2mM each of dNTPs, 0.5 µM of each primer, and 2 µl of DNA template. PCR was carried out with a MJ Research PT100 thermal cycler using the following conditions: 94 °C for 3 min; 94 °C for 30 sec, 64 °C for 30 sec and 72 °C for 1 min for 25 cycles; with a final extension at 72 °C for 7 min. PCR products were visualised on a 2 % agarose gel stained with ethidium bromide.

2.4.2. Nucleotide Sequence Analysis of AmpC Encoding Genes

Nucleotide sequencing of PCR amplicons was undertaken by dideoxy-chain termination method using the services of commercial vendor (Link Biotech, India). Sequence analysis and comparisons were performed using programs available at the NCBI server (http://www.ncbi.nlm.nih.gov).

2.5. Statistical Analysis

The performance of all the phenotypic tests was evaluated using PCR as a gold standard method. All the tests were repeated twice and the results were subjected to statistical analysis with Microsoft Excel XP (Microsoft, Seattle, WA) and SPSS version 10.0 software (SPSS Inc., Chicago, IL) and p-value was calculated using the χ² test. A value of p < 0.05 was considered statistically significant.

3. Results and Discussion

Isolates producing AmpC β-lactamases raise special concerns as these isolates have been responsible for several nosocomial outbreaks and high rate of clinical failure among infected patients has often been reported (5), (16) and (17). Till date, several phenotypic tests for the identification of AmpC producing isolates have been developed. However, there are presently no CLSI approved tests for identification of AmpC β-lactamase producing bacterial pathogens.

The result from the present study showed that of the total isolates collected, as many as 71/250 (28.4%) of *E. coli*, 64/199 (32.1%) of *K. pneumoniae* and 11/51 (21.5%) of *K. oxytoca* isolates showed an inhibition zone diameter of <18 mm and were positive for presumptive AmpC producers. Of these 71 screen positive isolates of *E. coli*, 29 (40.8%) were identified as AmpC producers by modified three dimensional test, AmpC disk test as well as with inhibitor based detection method. However, AmpC disk test with Tris-EDTA could identify 19/71 (26.7%) isolates of *E. coli* to be AmpC producers. On the other hand 22/64 (34.3%) isolates of *K. pneumoniae* and 2/11 (18.1%) of *K. oxytoca*, were found to be AmpC producers by modified three dimensional test and AmpC disk test whereas, inhibitor based detection method identified 23/64 (35.9%) of *K. pneumoniae* and 2/11 (18.1%) of *K. oxytoca* to be AmpC producers. However, in comparison to these tests AmpC disk test with Tris-EDTA detected 20/64 (31.2%) of *K. pneumoniae* and 1/11 (9%) isolate of *K. oxytoca* to be AmpC positive. (Table 1, Figure 1, 2, 3, 4).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Number of PCR positive isolates</th>
<th>Phenotypic tests</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M3DT</td>
<td>AmpC disk test</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>n = 71</td>
<td>25 (35.2)</td>
<td>29 (40.8)</td>
</tr>
<tr>
<td></td>
<td>ACT-1: 5</td>
<td>ACT-1: 5</td>
<td>ACT-1: 5</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>n = 64</td>
<td>20 (31.2)</td>
<td>22 (34.3)</td>
</tr>
<tr>
<td></td>
<td>ACT-1: 7</td>
<td>ACT-1: 7</td>
<td>ACT-1: 7</td>
</tr>
<tr>
<td><em>K. oxytoca</em></td>
<td>n = 11</td>
<td>2 (18.1)</td>
<td>2 (18.1)</td>
</tr>
<tr>
<td>Total</td>
<td>n = 146</td>
<td>47 (32.1)</td>
<td>53 (36.3)</td>
</tr>
</tbody>
</table>

n= number of isolates
Figures in parenthesis depict percentages
Detection and Characterization of AmpC β-Lactamases in Indian Clinical Isolates of Escherichia Coli, Klebsiella Pneumonae and klebsiella Oxytoca

A study showed M3DT and AmpC disk test to be equally sensitive in detecting AmpC producing gram negative bacteria (18). Whereas, another study which compared M3DT,AmpC disk test with Tris-EDTA, and the inhibitor based test, reported the inhibitor based test to be the most sensitive(19). Likewise, our study showed that inhibitor based detection method was 100% sensitive and 96% specific in detecting AmpC producers. (Table 2) Hence, Inhibitor based detection test being easier to perform and sensitive should be the choice.

<table>
<thead>
<tr>
<th>Phenotypic Tests</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
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</thead>
<tbody>
<tr>
<td>M3D test</td>
<td>100</td>
<td>91.30</td>
<td>86.21</td>
<td>100</td>
</tr>
<tr>
<td>AmpC disk test</td>
<td>100</td>
<td>91.30</td>
<td>86.21</td>
<td>100</td>
</tr>
<tr>
<td>AmpC disk test with Tris-EDTA</td>
<td>60.0</td>
<td>91.30</td>
<td>78.95</td>
<td>80.77</td>
</tr>
<tr>
<td>Inhibitor based detection test</td>
<td>100</td>
<td>91.30</td>
<td>86.21</td>
<td>100</td>
</tr>
</tbody>
</table>

Identification and characterization of AmpC β-lactamases is essential in order to follow the epidemiology of a specific AmpC type in a particular geographical area. A multiplex PCR which distinguished plasmid mediated AmpC enzymes from chromosomal AmpC β-lactamases and also differentiated between six different families of plasmid mediated AmpC enzymes in Klebsiellasp. and E. coli was developed by Perez and Hanson in 2002 (15).
In our study, PCR and nucleotide sequence analysis showed the presence of \textit{ampC} gene in 25 (35\%) (20-\textit{blaCMY-2}; 5-\textit{blaACT-1}) of the screen positive isolates of \textit{E. coli}, 20 (31.2\%) (13-\textit{blaCMY-2}; 7-\textit{blaACT-1}) of the screen positive isolates of \textit{K. pneumoniae} and 2 (18\%) (\textit{blaCMY-2}) of the screen positive isolates of \textit{K. oxytoca}. Several other studies from different parts of the world have reported the presence of these subtypes in isolates of \textit{E. coli} and \textit{Klebsiella spp.}. Our study showed CMY-2 subtype of AmpC \(\beta\)-lactamases to be the predominant type in clinical isolates of \textit{E. coli} and \textit{Klebsiella spp.} similar to various other studies shown elsewhere (4), (21), (23) and (24). However, the presence of ACT-1 subtype in these isolates cannot be disregarded.

AmpC \(\beta\)-lactamase producing \textit{E. coli} and \textit{K. pneumoniae} are being increasingly reported from many parts of the world (5), (6) and (25). In our study as many as 25/250 (10\%) isolates of \textit{E. coli} and 22/250 (8.8\%) of \textit{Klebsiella spp.} were AmpC positive by PCR. The present study showed much higher prevalence rates of AmpC producing \textit{E. coli} and \textit{Klebsiella spp.} than the ones ranging from 2\% to 7\% reported from various parts of the world (18), (26), (27) and (28). However, several other studies have reported much higher incidence ranging from 14-49\% of AmpC producing isolates of \textit{E. coli} and 17-58\% of \textit{Klebsiella} spp. (20), (29), and (30).

4. Conclusions

In the present study we compared four different phenotypic tests for the detection of AmpC producing isolates and found inhibitor based detection test to be the most sensitive and specific test. This test being easier to perform will be a helpful method for those who detect this enzyme routinely hence, we highly recommend this test. PCR and sequence analysis showed the presence of \textit{ampC} genes in the clinical isolates of \textit{E. coli} and \textit{Klebsiella spp.}. Our study showed 2 different subtypes of AmpC\(\beta\)-lactamases; CMY-2, which is the most common subtype of AmpC\(\beta\)-lactamases present all over the world and ACT-1 which is an inducible subtype, to be prevalent in clinical isolates of \textit{E. coli} and \textit{Klebsiella spp.} from different hospitals of Delhi region. Care should be taken in defining antibiotic policies where there is a presence of ACT-1 producing isolates as some of the antibiotics can induce the production of this particular subtype and hence, cause a therapeutic failure.

Our study showed an increased rate of prevalence of AmpC producing isolates of \textit{E. coli} and \textit{Klebsiella spp.} as compared to several other studies. It therefore, calls for a serious and concerted effort to rationalize the use of extended spectrum cephalosporins in order to contain this trend.

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