Prevalence of AMPC Beta Lactamases among Gram Negative Bacterial Isolates in a Tertiary Care Hospital

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ABSTRACT

Gram negative bacilli producing AmpC β-lactamases are often responsible for multidrug resistance that is not always detected in routine antimicrobial susceptibility tests as there are no Clinical Laboratory Standards Institute (CLSI) or other approved criteria for AmpC detection, this remains a serious clinical concern. Hence it is necessary to know the prevalence of AmpC mediated resistance in a hospital setting so as to enable the clinician to select the appropriate antibiotic regimens. An attempt was made to detect the prevalence of AmpC β-lactamases in gram-negative bacilli. This prospective study was conducted in the Department of Microbiology at VIMS & RC. A total of 256 Gram negative clinical isolates were obtained from various clinical samples from out patients and in patients admitted into various wards and intensive care units. All the isolates were identified based on the microscopy, the colony morphology and the biochemical reactions according to the standard guidelines. Antimicrobial Susceptibility Testing was performed by modified Kirby Bauer's disc diffusion method. AmpC screening was done using cefoxitin disk diffusion method, screen positives were phenotypically confirmed by disk approximation test, AmpC disk test and three-dimensional test (3D) 256 gram negative isolates were obtained from various clinical specimens. 84 were from the out patients and 172 from inpatients. E. coli was isolated more frequently (32%) followed by Klebsiella pneumoniae (21%), Acinetobacter species (14%), Proteus mirabilis (3%) and Citrobacter freundii (3%). 68% (174/256) of the isolates were cefoxitin resistant by disc diffusion method and 32% (82/256) were sensitive. Among 174 screen positive isolates Amp C was confirmed in 59% (103/174) of the isolates, 84 (82%) were from inpatients and 19 (18%) from out patients. Amp C production was predominant in Acinetobacter baumannii (61%), followed by Klebsiella pneumoniae (42%), Pseudomonas aeruginosa (41%), Citrobacter freundii (38%), E. coli (30%) and Proteus mirabilis (25%). All Amp C producing isolates were sensitive to imipenem, however high level resistance was demonstrated to the other antibiotics used. Isolates producing AmpC β-lactamases raise special concerns as these isolates have been responsible for several nosocomial outbreaks and high rate of therapeutic failure, identification of these enzymes may aid in hospital infection control and help the physician to restrict the indiscriminate use of antibiotics thus decreasing the selective pressure, which generates antibiotic resistance. This study highly recommends performing screening and phenotypic tests for AmpC detection in a routine diagnostic laboratory work, however sequencing and typing the strains may be required to give a better insight into the genetic relatedness and the molecular epidemiology of this resistance mechanism.

Keywords
AmpC β-lactamases, Phenotypically, Gram negative organisms, Cefoxitin resistance, Multidrug resistance
Introduction

The rapid emergence of antibiotic resistance among the hospital pathogens is a serious threat to the management of infectious diseases. Beta-lactam antibiotics are the most frequently used antimicrobials for the empirical therapy. Production of beta-lactamases is one of the strategies adopted by the bacteria to develop resistance to beta-lactam class of antibiotics (Col Naveen Grover et al., 2013). AmpC β-lactamases and/or extended spectrum β-lactamases (ESBLs) are of particular concern. AmpC β-lactamases are clinically significant because they may confer resistance to penicillins, cephalosporins, oxyimino-cephalosporins (e.g., ceftriaxone, cefotaxime, and ceftazidime), cephapirins (e.g., cefoxitin and cefotetan) and monobactams. AmpC β-lactamase activity is not affected by the ESBL inhibitor clavulanic acid. In the Ambler structural classification of β-lactamases AmpC enzymes belong to class C, while in the functional classification scheme of Bush et al., they were assigned to group 1. Genes for AmpC β-lactamases are commonly found on the chromosomes of several members of the family Enterobacteriaceae, including Enterobacter, Shigella, Providencia, Citrobacter freundii, Morganella morganii, Serratia marcescens, Escherichia coli, Pseudomonas aeruginosa and Acinetobacter sp. AmpC β-lactamase production is chromosome or plasmid mediated. Chromosomal AmpC genes are expressed constitutively at a low level. Some Enterobacteriaceae, such as Enterobacter species, Citrobacter spp., and Serratia spp., carry an inducible AmpC gene. In these cases, the gene is strongly induced by β-lactams, such as cefoxitin and imipenem, with expression mediated by the regulator AmpR. Mutations in the repressor gene AmpD may lead to overproduction of AmpC β-lactamases. Plasmid-based AmpC genes are expressed constitutively in most cases. However, some plasmid-carried AmpC genes, such as the DHA-1 gene, are inducible by β-lactams, with expression regulated similar to that of inducible chromosomal AmpC genes. All plasmid-carried AmpC genes are considered to be of significant clinical relevance. AmpC overproduction in addition to porin mutations of the outer membrane can reduce susceptibility to carbapenems, in particular in plasmid mediated AmpC producers. Porins are chemically selective and transport only one group of molecules, or may be specific for one molecule. β-lactam and fluoroquinolone antibiotics must pass through porins to reach their targets in gram-negative bacteria. Bacteria can develop resistance to these antibiotics by mutating the gene that encodes the porin – the antibiotics are then excluded from passing through the outer membrane. Although reported with increasing frequency in case isolates, the true rate of occurrence of plasmid mediated AmpC β-lactamases in Klebsiella pneumoniae, E. coli and Proteus mirabilis remains unknown. These organisms usually exhibit multidrug resistance that is not always detected in routine antimicrobial susceptibility tests as there are no Clinical Laboratory Standards Institute (CLSI) or other approved criteria for AmpC detection (El-Hady and Adel, 2015; Ladhawa Avinash et al., 2012). It is necessary to know the prevalence of AmpC mediated resistance in a hospital setting so as to enable the clinician to select the appropriate antibiotic regimens. With this background this study was undertaken to detect the prevalence of AmpC β-lactamases in gram-negative bacilli at our institute.

Materials and Methods

This prospective study was conducted in the Department of Microbiology at VIMS & RC. A total of 256 clinical isolates (E. coli (n=82), Klebsiella pneumoniae (n=69),
**Pseudomonas aeruginosa** (n=54), **Acinetobacter baumannii** (n=36), **Proteus mirabilis** (n=8), **Citrobacter freundii** (n=8) were obtained from various clinical samples such as urine, pus, sputum, tracheal aspirate, catheter tip, aspirated fluids and blood from out patients and in patients admitted into various wards and intensive care units.

**Processing of samples**

All the samples were aseptically inoculated on to Blood and Mac Conkey agar plates and incubated overnight at 37°C. Urine was inoculated on hichrome (urochrome media). All the isolates were identified based on the colony morphology and biochemical reactions according to the standard guidelines (Koneman et al., 2006).

**Antimicrobial susceptibility testing**

Routine disc diffusion susceptibility testing was performed by modified Kirby Bauer’s disc diffusion method using discs of standard potency.

Cefotaxime-30 μg; ceftazidime-30 μg; ceftriaxone-30 μg; cefoxitin (30), Amikacin-10 μg; amoxyccillin-20 μg; gentamycin-10 μg; Tetracycline-30 μg; imipenem-30 μg; ciprofloxacin-5 μg; Aztreonam-30 μg; cotrimoxazole-1.25/23.75 μg. The results were interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI).

**AmpC screening using the disk diffusion method** (Mohamudha Parveen et al., 2010; Supriya Upadhyay et al., 2010)

Cefoxitin resistant strains were detected using cefoxitin disk 30 μg. Isolates with zone diameters less than 18 mm were considered AmpC screening positive and were selected for phenotypic AmpC confirmatory tests.

**Phenotypic AmpC confirmation methods**

**Disk approximation test** (El-Hady and Adel, 2015; Supriya Upadhyay et al., 2010; Michael Dunne and Hardin, 2005)

Used for the detection of inducible AmpC production. A test isolate (with a turbidity equivalent to that of 0.5 McFarland standards) was spread over a Mueller Hinton agar plate. Cefotaxime (30μg) and cefoxitin (30μg) disks were placed 20 mm apart from centre to centre. Isolates showing blunting of the cefotaxime zone of inhibition adjacent to the cefoxitin disk were screened as positive for AmpC β-lactamase (Figure 1).

**AmpC disk test** (Avinash et al., 2012; Coudron et al., 2003)

Used for the detection of plasmid mediated AmpC production. 0.5 McFarland suspension of ATCC E. coli 25922 was inoculated on the surface of Mueller-Hinton agar plate. A 30 μg cefoxitin disc was placed on the inoculated surface of the agar. A sterile plain disc inoculated with several colonies of the test organism was placed beside the cefoxitin disc almost touching it, with the inoculated disk face in contact with the agar surface. After overnight incubation at 37°C, the plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cefoxitin (positive result) or the absence of a distortion, indicating no significant inactivation of cefoxitin (negative result) (Figure 2).

**Three-dimensional test (3D)** (Gude et al., 2012; Coudron et al., 2000)

Plasmid mediated AmpC production was further confirmed by this method. This method is a modified Hodge test described by Coudron et al. (2000). A lawn of E. coli ATCC 25922 was inoculated on a Mueller-
Hinton agar plate. After the agar surface dried, a 30-µg cefoxitin disk was placed at the center and 20 µl of a 0.5 McFarland suspension of the test isolate was dispensed into a radial slit performed in the plate. After overnight incubation at 37°C, enhanced growth of the surface organism in the inhibition zone along with the test strain was interpreted as evidence for the presence of AmpC β-lactamase (Figure 3).

**Results and Discussion**

In this study 256 gram negative isolates were obtained from various clinical specimens such as urine (n=97), pus (n=56), sputum (n=38), tracheal aspirate (n=29), aspirated fluids (n=18), catheter tip (n=13) and blood (n=5).

Among the 256 gram negative isolates 84 were from the out patients and 172 from inpatients.

*E. coli* was isolated more frequently (32%) followed by *Klebsiella pneumoniae* (27%), *Pseudomonas aeruginosa* (21%), *Acinetobacter baumannii* (14%), *Proteus mirabilis* (3%) and *Citrobacter freundii* (3%).

Of the 256 gram negative isolates 68% (174/256) were deemed cefoxitin resistant by disc diffusion method and 32% (82/256) were sensitive.

Among 174 screen positive isolates Amp C was confirmed by disc antagonism test, AmpC disk test and Three-dimensional test in 59% (103/174) of the isolates (Table 1).

Of the 103 Amp C positive isolates 84 (82%) were from inpatients and 19 (18%) from out patients.

Highest percentage of Amp C was reported from pus (61%), followed by tracheal aspirate isolates (48%), aspirated fluids (44%), catheter tip (38%), urine (36%), blood (20%) and sputum (16%) (Table 2).

Amp C production was predominant in *Acinetobacter baumannii* 22/36 (61%), followed by *Klebsiella pneumoniae* 29/69 (42%), *Pseudomonas aeruginosa* 22/54 (41%), *Citrobacter freundii* 3/8 (38%), *E. coli* 25/82 (30%) and *Proteus mirabilis* 2/8 (25%) (Table 3).

All Amp C producing isolates were sensitive to imipenem, however high level resistance was demonstrated to the other antibiotics used.

Drug resistance poses a therapeutic problem not only in the hospital settings, but also in the community as most of the bacteria have acquired resistance to multiple antibiotics. Antimicrobial drug resistance is emerging world wide as a major public health problem. Selective pressure of misuse and overuse of antibiotics in the hospitals has resulted in the emergence and dissemination resistant bacteria in many areas of hospitals. The various mechanisms of drug resistance in Gram negative bacteria include extended spectrum b-lactamases (ESBL) production, AmpC β-lactamase production, efflux mechanism and porin deficiency. In the clinical laboratory settings, the commonly detected enzymes causing resistance are AmpC β-lactamases and ESBLs. Clinical relevance of AmpC β-lactamases lies in the fact that they confer resistance to both narrow and broad spectrum cephalosporins, β lactam/β lactamase inhibitor combinations and aztreonam. Detection of AmpC is important to improve the clinical management of patients suffering from infections and would also provide us with sound epidemiological data. However, there are no clinical and laboratory standard institute guidelines for detection of AmpC mediated resistance in gram negative...
clinical isolates and hence, it usually poses a problem due to misleading results, especially so in phenotypic tests (Col Naveen Grover et al., 2013; El-Hady and Adel, 2015).

In this study, sizeable number of cefoxitin resistant isolates was not positive for AmpC production. Among 174 screen positive isolates Amp C was confirmed phenotypically in 59% (103/174) of the isolates, such a finding was also seen by Anand Manoharan et al. (2012) wherein only 36.5% cefoxitin resistant isolates were confirmed Amp C producers phenotypically, this warrants further investigation into the other mechanisms of resistance, this could be due to the inability of current phenotypic tests to accurately detect the type of transferable AmpC β lactamase which does not allow for the differentiation of multiple AmpC enzymes and it is possible that there is a limit to the amount of AmpC β lactamase that a bacterial cell can accommodate and still be a viable pathogen. The available tests are inadequate for organisms expressing plasmid-mediated AmpC β lactamases alone or in combinations with ESBLs. However the use of cefoxitin resistance as a screening agent/marker for AmpC production is quite reliable. The use of phenotypic methods in combination with cefoxitin as screening method may be a better tool for laboratory diagnosis and confirmation of AmpC producing Gram-negative bacteria (Anand Manoharan et al., 2012).

In India the incidence of Amp C production among clinical isolates has been on steady increase over the past few years and ranges from 3.3–47% as seen by various authors, in the present study 40% were Amp C producers which is in accordance with the Indian scenario. Despite the phenotypic methods used, we were not able to distinguish between the chromosomal derepressed and plasmid mediated enzymes as this requires isoelectric focusing and genotypic methods (Col Naveen Grover et al., 2013).

The high percentage of AmpC harbouring isolates was encountered in isolates from inpatients, showing its nosocomial importance. 61% isolates of pus, 48% of tracheal aspirate, 44% of aspirated fluids, and 38% of catheter tip isolates harboured AmpC enzymes reinforcing the fact that there is rampant spread of antibiotic resistance among the hospital strains (Upadhyyay et al., 2013; Varsha Gupta et al., 2012).

Emerging evidence suggests that probably all Acinetobacter baumannii isolates produce a chromosomal Amp C enzyme. In the present study 61% of Acinetobacter baumannii isolates were found to harbour an Amp C enzyme. The reason for low occurrence could be that all the isolates may have AmpC genes, but these might not be expressed in all the isolates of Acinetobacter baumannii, they might have ‘silent genes’ or there might be low level expression of AmpC genes that was not detected by the present method (Vikas Manchanda and Singh, 2003). 41% of Pseudomonas aeruginosa isolates had Amp C enzymes as against high prevalence reported by Supriya Upadhyay et al. (2010) and Şerife Altun et al. (2013).

Pseudomonas aeruginosa may be intrinsically resistant or has acquired resistance to antibiotics due to permeability barrier of the cell surface, multidrug efflux pumps and production of β lactamases (AmpC β lactamase, extended spectrum β lactamases and metallo β lactamases). Michael Dunne and Hardin (2005) detected AmpC production in 77% isolates in Citrobacter species, Citrobacter freundii.
having the highest percentage, *Citrobacter freundii* possess inducible AmpC β lactamases (AmpC) that can be upregulated by subinhibitory concentrations of certain β lactam antibiotics.

**Table.1** Prevalence of Amp C in cefoxitin resistant cases

<table>
<thead>
<tr>
<th>Total no of isolates</th>
<th>Cefoxitin Sensitive</th>
<th>Cefoxitin Resistance</th>
<th>Amp C %</th>
</tr>
</thead>
<tbody>
<tr>
<td>256</td>
<td>82</td>
<td>174</td>
<td>103/174 (59%)</td>
</tr>
</tbody>
</table>

Of the 256 gram negative isolates 82 were cefoxitin sensitive and 174 were resistant. Of the 174 isolates 103 (59%) were AmpC producers.

**Table.2** Prevalence of Amp C beta lactamase in various clinical samples

<table>
<thead>
<tr>
<th>S.l no</th>
<th>Sample</th>
<th>n</th>
<th>Amp C %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Urine</td>
<td>97</td>
<td>35      (36%)</td>
</tr>
<tr>
<td>2</td>
<td>Pus</td>
<td>56</td>
<td>34      (61%)</td>
</tr>
<tr>
<td>3</td>
<td>Sputum</td>
<td>38</td>
<td>6       (16%)</td>
</tr>
<tr>
<td>4</td>
<td>Tracheal aspirate</td>
<td>29</td>
<td>14      (48%)</td>
</tr>
<tr>
<td>5</td>
<td>Aspirated fluids</td>
<td>18</td>
<td>8       (44%)</td>
</tr>
<tr>
<td>6</td>
<td>Catheter tip</td>
<td>13</td>
<td>5       (38%)</td>
</tr>
<tr>
<td>7</td>
<td>Blood</td>
<td>5</td>
<td>1       (20%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>256</strong></td>
<td><strong>103</strong> (40%)</td>
</tr>
</tbody>
</table>

Highest percentage of Amp C was reported from pus (61%), followed by tracheal aspirate isolates (48%), aspirated fluids (44%), catheter tip (38%), urine (36%), blood (20%) and sputum (16%)

**Table.3** Production of Amp C beta lactamase in various organisms

<table>
<thead>
<tr>
<th>Sl. no</th>
<th>Organism</th>
<th>No of isolates</th>
<th>Amp C %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>E. coli</em></td>
<td>82</td>
<td>25      (30%)</td>
</tr>
<tr>
<td>2</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>69</td>
<td>29      (42%)</td>
</tr>
<tr>
<td>3</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>54</td>
<td>22      (41%)</td>
</tr>
<tr>
<td>4</td>
<td><em>Acinetobacter baumannii</em></td>
<td>36</td>
<td>22      (61%)</td>
</tr>
<tr>
<td>5</td>
<td><em>Proteus mirabilis</em></td>
<td>8</td>
<td>2       (25%)</td>
</tr>
<tr>
<td>6</td>
<td><em>Citrobacter freundii</em></td>
<td>8</td>
<td>3       (38%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>256</strong></td>
<td><strong>103</strong> (40%)</td>
</tr>
</tbody>
</table>

Among the gram negative isolates Amp C production was predominant in *Acinetobacter baumannii* (61%), followed by *Klebsiella pneumoniae* (42%), *Pseudomonas aeruginosa* (41%), *Citrobacter freundii* (38%), *E. coli* (30%) and *Proteus mirabilis* (25%).
Isolates showing blunting of the cefotaxime zone of inhibition adjacent to cefoxitin disk are AmpC β-lactamase producers.

AmpC disk test showing distortion of the inhibition zone around cefoxitin disk (CX) in the vicinity of the disk containing the test organism, indicating a positive test.

Organism showing clear distortion in the zone of inhibition strain 1 (positive control), 2 & 3 (test strains) and showing minimal distortion are AmpC producers and no distortion strain 4 (negative control) indicates non-AmpC producers.

Further, mutations can occur in the regulatory components of AmpC leading to a stable, hyperproduction of AmpC with concomitant high-level resistance to many classes of β-lactam antibiotics, however in the present study 3/8 (38%) of Citrobacter
*freundii* had Amp C production, low prevalence could be due the low sample size, genetic level study could have possibly explained this ambiguity.

AmpC β-lactamase producing *E. coli* and *Klebsiella pneumoniae* are being increasingly reported from many parts of the world incidence ranging from 14-49% for *E. coli* and 17–58% for Klebsiella spp. In the present study the percentage of Amp C production in *E. coli* and *Klebsiella pneumoniae* was 30% and 42% respectively which is in accordance to the previous studies (Tanushree Barua et al., 2013). The drug of choice remains carbapenems in Amp C producers as the β-lactam / β-lactamases inhibitor combinations fail even if these show sensitivity in vitro, the present study showed 100% sensitivity to imipenem

In conclusion, AmpC β-lactamase producing bacterial pathogens may cause a major therapeutic failure if not detected and reported in time AmpC β-lactamase. Identification of AmpC may aid in hospital infection control and help the physician to prescribe the most appropriate antibiotic, thus decreasing the selective pressure, which generates antibiotic resistance. Sequencing and typing the strains may be required to better understand the genetic relatedness and the molecular epidemiology of this resistance mechanism.

**Conflict of interest:** The authors declare that there are no conflicts of interest

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