**Inducible and Plasmid Mediated AmpC Beta-Lactamase among *Klebsiella pneumoniae* in a Tertiary Care Teaching Hospital of South India**

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**ABSTRACT**

The incidence of AmpC beta-lactamase producing *K. pneumoniae* has been steadily increasing over past years and is of significant concern as they restrict therapeutic options and cause treatment failure. The objective of the present study was to know the occurrence of AmpC beta-lactamase producing *K. pneumoniae* and to create a baseline antibiotic resistance data to implement effective antibiotic policy. A total of 132 non duplicate *K. pneumoniae* isolates obtained from various clinical samples over a period of 18 months were tested for inducible and plasmid mediated AmpC beta-lactamases by disc antagonism and AmpC disc test respectively. 11.3% were inducible and 28.7% were plasmid mediated AmpC beta-lactamases producers. Detection of inducible and plasmid mediated Amp C beta-lactamases was higher among outpatient (18.7% and 34.3%) compared to ward isolates (10.3% and 29.3%) and ICU isolates (7.1% and 23.8%). MDRs among inducible and plasmid mediated beta-lactamases were 20% and 44.7% respectively. All the MDRs with Amp C beta-lactamase producers were susceptible to cefipime and imipenem. Association of aminoglycosides and imipenem resistance among Amp C beta-lactamase positive isolates was higher compared to negative isolates (*p* value < 0.5) whereas cefoperazone-sulbactam, piperacillin-tazobactam, cefipime, ciprofloxacin and co-trimoxazole resistance did not show significant association (*p* value > 0.5) among positive and negative isolates. Cefipime and imipenem resistance among cefoxitin resistant isolates was 81.8% and 39.3% respectively. Occurrence of these enzymes associated with antibiotic resistance and the clinical implications should be cautiously considered during the establishment of an antibiotic policy in a hospital setting.

**Keywords**

*Klebsiella pneumoniae*, Inducible and Plasmid mediated AmpC-β lactamases, Multi-drug resistance, Teaching hospital.

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**Introduction**

Amp C beta-lactamases are a type of cephalosporinase enzymes belonging to Class C of Ambler’s structural classification, while assigned to group 3 in the functional classification scheme of Bush (Ambler, 1980; Bush *et al.*, 1995). These enzymes can be either chromosomal or plasmid encoded conferring resistance to both oxyimino (cefotaxime, ceftazidime, ceftriaxone) and 7-α methoxy cephalosporins (cefoxitin or cefotetan) and are not affected by β-lactamase inhibitors (clavulanate, sulbactam, tazobactam) (Phillipon *et al.*, 2002). Plasmid mediated Amp C β-lactamases are not
inducible in contrast to chromosomal which can be induced and are associated with multidrug resistance (MDR). The emergence and rapid spread of MDR \textit{Klebsiella pneumoniae} isolates is becoming a serious antibiotic management problem causing a great concern worldwide due to these enzymes (Jemima \textit{et al.}, 2008; Acheampong \textit{et al.}, 2011). Differentiating AmpC positives from negative isolates would prevent the unnecessary usage of cephalosporins and carbapenems resulting in the selective pressure driving the AmpC or plasmid mediated class A carbapenem resistance gene propagation (Hanson, 2003). There are also concerns that treatment failures will occur with certain cephalosporins due to incorrect susceptibility tests when organisms are enzyme positive but appear falsely susceptible. Hence, detection of such enzymes among clinical isolates becomes important to safeguard successful therapeutic intervention and beneficial clinical outcome (Black \textit{et al.}, 2005; Hernandez-Alles \textit{et al.}, 1999). The present study was undertaken to know the occurrence of Amp C – βlactamases among \textit{K pneumoniae} and their association with various antibiotic groups at a tertiary care teaching hospital.

**Materials and Methods**

The study was conducted between November 2012 to April 2014 at ESIC-MC and PGIMSR, Rajajinagar, Bengaluru, a 500 bed tertiary care teaching hospital. \textit{K pneumoniae} isolates from various clinical samples like pus, urine, sputum, blood, miscellaneous (throat swabs, vaginal swabs, body fluids) from both out-patients and inpatients submitted to diagnostic microbiology were included. \textit{K pneumoniae} were isolated (one isolate per patient) and identified by standard methods. Antibiotic susceptibility test was performed by Kirby- Bauer’s disc diffusion method using commercially available antibiotic discs (Hi Media, Mumbai, India).

Ceftazidime (30μg), ceftazidime/ clavulanicacid (30/10μg ), cefoxitin (30μg), cefotaxime (30 μg), cefoperazone (75μg), cefoperazone (75 μg)/ sulbactum (10μg), ceftriaxone (30 μg), ciprofloxacin (5μg), amikacin (30 μg), amoxicillin/ clavulanic acid (20/10μg), aztreonam (30μg), ceftipeime (30μg), imipenem (10μg), piperacillin (100 μg), piperacillin/tazobactam (100/10μg), gentamicin (10μg), trimethoprim/ sulphamethoxazole (1.25/23.75μg).

Isolates resistant to cefoxitin (<18 mm zone diameter), third generation cephalosporins (3 GC), and negative for ESBLs by double disc synergy test (cephalosporin / clavulanic acid) were tested for inducible AmpC beta-lactamases by disc antagonism test \textsuperscript{11} and for plasmid mediated AmpC beta-lactamases by AmpC disk test (Black \textit{et al.}, 2005).

**Disc antagonism test:** Briefly, a disc of cefoxitin (30μg) and other beta lactam discs (cefotaxime, cefaperazone, ceftazidime) were placed at a distance of 25 mm apart on a lawn culture of test isolate on MHA plate and incubated overnight at 37 °C. If radius is smaller by 4 mm or more of any 3 GCs towards cefoxitin disc, antagonism was considered and was labeled as positive (Fig 1).

**AmpC disk test:** Briefly, 0.5 McFarland suspension of \textit{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 surface facing the agar. The plate was then inverted and incubated over night at 37°C. After incubation, plates were examined for either an indentation or a flattening of the zone of inhibition, indicating enzymatic inactivation of cefoxitin (Amp C producers), or the absence of a distortion,
indicating no significant inactivation of cefoxitin (Amp C non producers) (Fig 2).

**Statistical analysis**

Data was entered into Microsoft Excel to calculate percentage of antibiotic resistance. Statistical significance of association between AmpC beta-lactamase producers to other antibiotics was assessed by calculating P value using Chi-square test.

**Results and Discussion**

Out of 132 cefoxitin resistant isolates, 11.3% (15/132) were inducible and 28.7% (38/132) were plasmid mediated AmpC beta-lactamases producers. Detection of inducible and plasmid mediated Amp C beta-lactamases was higher among outpatient isolates (18.7% and 34.3%) compared to ward (10.3% and 29.3%) and ICU isolates (7.1% and 23.8%) (Table 1). MDR among inducible and plasmid mediated AmpC producers was 20% (3/15) and 44.7% (17/38) respectively with overall MDRs of 37.7% (20/53). All 20 isolates of AmpC producing MDRs were susceptible to cefipime and impenem. Association of gentamicin, amikacin and imipenem resistance among Amp C beta-lactamase positive isolates were higher compared to Amp C beta-lactamase negative isolates (p value < 0.5) whereas cefoperazone-sulbactam, piperacillin- tazobactam, cefipime, ciprofloxacin and co-trimoxazole resistance did not show significant association (p value > 0.5) among positive and negative isolates (Table 2). Overall cefipime and imipenem resistance among cefoxitin resistant isolates was 81.8% (108/132) and 39.3% (52/132) respectively. There are various types of plasmid-mediated AmpC β-lactamases: CMY, MIR, MOX, LAT, FOX, DHA, ACT, ACC, and CFE reported worldwide (Jacoby, 2009; Dunne et al., 2000). First report of plasmid mediated AmpC β-lactamase from *K. pneumoniae* isolates was from Seoul, South Korea in 1989. In India, AmpC producing strains of Enterobacteriaceae of type CMY-4 and CMY-6 were reported during 1998 and 2009 respectively (Philippon et al., 2002). In the present study 28.7% of the isolates were found to be plasmid mediated AmpC producer which is higher compared to other studies from India. Study from Chennai during 2005 reveals 20.8% AmpC producers whereas study from Bangalore during 2012 reveals 7.7% as AmpC producers among *K pneumonia* (Sasirekha et al., 2012). Studies also reveal a relatively higher percentage of these enzymes when compared to earlier report of 2.2% for *K pneumoniae* isolates in Karnataka (Ratna et al., 2003). AmpC genes in *K pneumoniae* ranged from as low as 0.4% from Switzerland to as high as 77% from Korea (Yong et al., 2005). Overall there is an increasing trend in the prevalence of these enzymes over a period of time threatening the existing limited antibiotic spectrum.

Risk associated with these enzymes that plasmids carrying genes for AmpC beta-lactamases often carry multiple other resistances including genes for resistance to aminoglycosides, chloramphenicol, quinolones, sulfonamide, tetracycline, and trimethoprim as well as genes for other beta-lactamases. The prevailing hypothesis of MDR *K pneumoniae* is that these bacteria acquire MDR through horizontal transfer of antimicrobial resistance genes mediated by mobile genetic elements such as integrons. These MDR strains co-carrying diverse and numerous multiple resistance determinants may impose limitations in the therapeutic options available for the treatment of infections (Cao et al., 2014). Various studies from India have reported high prevalence (as high as 87.6%) MDR phenotype among clinical isolates of *K. pneumonia* (Bora et al., 2014; Datta et al., 2012). The present study show 37.7% MDRs among AmpC beta-
lactamase producers and statistically reveals AmpC beta-lactamase producers are likely to be associated with resistance to aminoglycosides and carbapenems than the non-producers, possibly indicating gene carrying multiple resistances that needs further study to understand this association in the present clinical setting. Present study also documents a higher AmpC beta-lactamase producer among outpatient than inpatients similar to Gude et al. who found higher resistance rate among community patients. Food products (Ahmed et al., 2009) drinking water and river beaches (Mataseje et al., 2009) have been identified as risk factors for community based sources of AmpC producers which pose a serious risk of transmission to hospitalized patients when infected or colonized patients are admitted. This emphasizes early detection and prevention of spread of such resistant isolates in hospitals with appropriate infection control measures. Treatment options are severely limited due to associated multiple genes of resistance and beta-lactamases. Fourth generation cephalosporins and carbapenems have been recommended for the treatment of infections caused by AmpC-producing strains of *K. pneumonia* (Shi et al., 2009; Queenan, 2007).

Table 1 Distribution of Amp C beta lactamases resistance of *K. pneumoniae* in different patient category

<table>
<thead>
<tr>
<th>Patient Category</th>
<th>Inducible AmpC-β lactamases</th>
<th>Plasmid mediated AmpC-β lactamases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Out Patient (n=32)</td>
<td>6 (18.7)</td>
<td>11 (34.3)</td>
</tr>
<tr>
<td>wards (n=58)</td>
<td>6 (10.3)</td>
<td>17 (29.3)</td>
</tr>
<tr>
<td>Intensive care Unit(n=42)</td>
<td>3 (7.1)</td>
<td>10 (23.8)</td>
</tr>
<tr>
<td>Total (n=132)</td>
<td>15 (11.3)</td>
<td>38 (28.7)</td>
</tr>
</tbody>
</table>

Table 2 Comparison of antibiotic resistance pattern among Amp C positive and negative *Klebsiella pneumoniae* isolates

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>AmpC beta-lactamase</th>
<th>Z-value (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Positives N = 53</td>
<td>No. of Negatives N= 79</td>
</tr>
<tr>
<td>Cefoperazone-sulbactam</td>
<td>14 (26.4)</td>
<td>33 (41.7)</td>
</tr>
<tr>
<td>Piperacillin- tazobactam</td>
<td>15 (28.3)</td>
<td>33 (41.7)</td>
</tr>
<tr>
<td>Cefipime</td>
<td>15 (28.3)</td>
<td>26 (32.9)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>17 (32.0)</td>
<td>40 (50.6)</td>
</tr>
<tr>
<td>Amikacin</td>
<td>20 (37.7)</td>
<td>52 (65.8)</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>17 (32.0)</td>
<td>26 (32.9)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>17 (32.0)</td>
<td>32 (40.5)</td>
</tr>
<tr>
<td>Imipenem</td>
<td>29 (54.7)</td>
<td>63 (79.7)</td>
</tr>
</tbody>
</table>

Numbers in parenthesis are resistance percentage
S: Significant (p value<0.05), NS: Non significant (p value >0.05)
**Fig.1** Disc antagonism method positive for inducible Amp C-β lactamase (blunting of 3GC disc zone adjacent to cefoxitin disc

CX: cefoxitin, CTX: cefotaxime, CAZ: ceftazidime, CPZ: cefoperazone

**Fig.2** AmpC disc test for plasmid mediated Amp C-β lactamase detection

Three Positives: blunting towards cefoxitin disc (CN)
One Negative: No blunting towards cefoxitin disc

Cefepime, a fourth generation cephalosporin is a weak inducer of AmpC beta-lactamase, quickly enter through the outer cell membrane, and is hydrolyzed less by the enzyme (Sanders, 1993). Several AmpC-producing organisms test cefepime susceptible with a conventional inoculum but express resistance with higher inoculum concentration suggesting caution in its use. Besides, recommendation of cefepime to be an exception to avoid all cephalosporin therapy even if an AmpC-producing isolates tests susceptible to an individual agent also remains debatable. All the AmpC producers in the present study were susceptible to cefipime but overall resistance among cefoxitin resistant isolates was higher (81.8%). Hence clinical utility and decision in using cefipime depends on the suggestions made on the basis of specific cephalosporin MICs rather than on the presence of AmpC beta-lactamase enzyme (Goldstein, 2002) underlining the need for low resourceful laboratories to perform MIC at least on resistant isolates. Inducible expression of chromosomal Amp C beta-lactamases,
although rare in *K. pneumoniae*, also associated with a significant risk of therapeutic failure with all beta-lactam drugs except carbapenems. Present study reveals 11.3% inducible beta-lactamase producers, whether these isolates were plasmid or chromosomal to be ascertained by further molecular study. Even though carbapenems remains best choice to treat infections due to AmpC-producing bacteria, 39.3% of imipenem resistance among non AmpC producers in the present study warns its judicious use and demands to look out for possible *K pneumoniae* carbapenemases. It would be prudent also to caution the clinicians when the presence of such resistant isolates are suspected or detected, and to prevent the use of strong AmpC inducing agents, such as clavulanic acid and cephamycins (Jing-Jou *et al.*, 2002).

The present preliminary study was based on phenotypic tests which do not differentiate between the plasmid-mediated enzymes producers and the chromosomal hyper producers or porin loss mutants. In addition, possible mechanism of resistance among isolates with cefoxitin and imipenem resistance (carbapenemase) with non AmpC producers could not be established. Further studies are needed to know the other resistance mechanisms, confirmation and differentiation of these enzymes by molecular methods.

In conclusion, Inducible and plasmid mediated Amp C beta-lactamases with MDR were found to be prevalent. Higher isolation rate of these enzymes from out patients probably indicates community source or previous hospitalization as risk factors. Statistical significance of association of these enzymes with aminoglycosides and carbapenems as well as non AmpC producers needs further study. Susceptibility of these enzymes to cefipime and imipenem and their clinical utility needs continuous monitoring and surveillance during the establishment of an antibiotic policy in a hospital setting.

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