Diagnostic utility of boronic acid inhibition with different cephalosporins against *Escherichia coli* producing AmpC β-lactamases

*Escherichia coli*, one of the most important bacterial pathogens capable of causing community and nosocomial infections, may become resistant to cefamycins and oximino-cephalosporins by virtue of promoter and attenuator mutations or because they have acquired mobilized β-lactamas from other Gram-negative bacilli (Jacoby, 2009). The chromosomally encoded AmpC β-lactamase of *E. coli* is non-inducible, because of the absence of the regulatory gene *ampR*; the constitutive low level expression of chromosomal AmpC does not contribute to a clinically relevant level of resistance to β-lactams (Honore *et al.*, 1986). However, hyperproduction of these enzymes and plasmid-encoded AmpC enzyme can cause resistance to penicillins, first and second generation cephalosporins, cephapemycin and to oximino-cephalosporins (Doi & Paterson, 2007). The methods for detecting AmpC hyperproduction or plasmid-encoded AmpC enzymes among *E. coli* are technically demanding for clinical laboratories.

The detection of AmpC enzymes in Gram-negative organisms poses a serious problem due to misleading results. To date, no Clinical and Laboratory Standards Institute (CLSI) guidelines or other approved criteria have been developed for the detection of AmpC in enterobacteriaceae (Fernández-Cuenca *et al.*, 2005). In numerous studies conducted worldwide, the modified three dimensional test (M3DT) and multiplex PCR assay have been considered as gold standards for the detection of AmpC (Coudron 2005; Pérez-Pérez & Hanson, 2002). But it was found that both of these are time consuming and labour intensive methods; thus, they cannot be used in the routine clinical laboratory. A routine clinical microbiology laboratory requires a simple, rapid and cost effective method to screen AmpC β-lactamases among clinical isolates to enable implementation of proper antimicrobial therapy. Boronic acid has long been considered as an AmpC inhibitor and the boronic acid test has an enhanced detection capability among enterobacterial isolates producing AmpC β-lactamase (Coudron, 2005). Therefore, the aim of the current study was to evaluate the combination of three different cephalosporins along with boronic acid as an AmpC inhibitor for the detection of AmpC β-lactamase enzyme among clinical isolates of *E. coli*.

In the study, a total of 455 consecutive clinical isolates of *E. coli* were collected from inpatients and outpatients of Sir Sunder Lal Hospital of Banaras Hindu University, Varanasi, India, during April 2008 to September 2009. The isolates were identified by standard biochemical tests (Colee *et al.*, 1996). A previously confirmed clinical isolate of *E. coli* harbouring *blaCTX* was used as positive control and *E. coli* ATCC 25922 was used as negative control.

Screening for AmpC β-lactamase producers was performed by cefoxitin (30 μg) disc test. Isolates that yielded a zone diameter less than 18 mm were suspected to be AmpC producers and further subjected to confirmatory testing. All the screened positive isolates were tested by M3DT as described by Coudron *et al.* (2000). The test was performed by standard disc diffusion method, placing 30 μg cefoxitin, ceftazidime and cefotaxime (Hi-Media, Mumbai, India) discs alone and in combination with 400 μg boronic acid (Hy-Glass and Chemicals) onto Mueller–Hinton agar (Hi-Media). After overnight incubation, an organism that demonstrated a zone diameter of 5 mm or greater around the disc of cephalosporin plus boronic acid than the zone diameter of cefepirin alone was considered as an AmpC β-lactamase producer (Coudron, 2005). For better evaluation, 10 previously confirmed extended-spectrum β-lactamase (ESBL) positive but AmpC negative isolates with elevated cephalosporin MICs were also used for the study. The ESBL status of these strains was established by combined disc diffusion method as per CLSI recommendations (CLSI, 2005) using cefotaxime (30 μg) and ceftazidime (30 μg) discs alone and in combination with clavulanic acid.

As KPC β-lactamase also shows inhibition by clavulanic acid and boronic acid, the presence of KPC can be misinterpreted as co-production of ESBL and AmpC; to rule out that possibility, the status of KPC was determined by both boronic acid and clavulanic acid inhibition test, as well as by modified Hodge test as described by Lee *et al.* (2001).

The MICs for all the isolates were tested by the agar dilution method according to CLSI recommendations (CLSI, 2005) for antibiotics cefotaxime, ceftazidime, ceftriaxone (Hi Media), cefepime (Alembic), aztreonam (Aristo Pharmaceuticals), imipenem (United Biotech) and meropenem (Astra Zeneca Pharmaceuticals). *E. coli* ATCC 25922 was used as a control.

DNA was extracted by the boiling centrifugation method. For partial gene PCR amplification, primers specific for *blaMOX, blaCTX, blaDHA, blaACC, blaTBC* and *blaFOX* genes were used as described by Pérez-Pérez & Hanson (2002). Random amplification of polymorphic DNA analysis was performed using primer 7 (5’-GTGGATGCGA-3’) and the isolates were typed according to their band patterns (Van Belkum *et al.*, 1995).

The M3DT was taken as the gold standard for measuring the efficiency of combinations. The diagnostic accuracy was determined, and the sensitivity and
specificity were also evaluated. Data were coded, entered and analysed by using SPSS (version 16) and presented as percentages.

AmpC resistance in E. coli can be due to the overexpression of the chromosomal ampC gene, acquisition of a plasmidic AmpC, alterations in the permeability of the cell to cefoxitin or a combination of these factors (Mulvey et al., 2005). As there is no standardized phenotypic method for screening and detection of this AmpC-mediated resistance, the surveillance and characterization of strains becomes difficult. Therefore, the current study focused on finding the best possible combination of cephalosporins along with boronic acid, as an AmpC inhibitor, that can screen the maximum AmpC-producing isolates.

Out of 455 E. coli isolates tested, cefoxitin resistance was evident in 345 (75.8 %). Of them 294 (64.6 %) exhibited AmpC activity by M3DT. In the inhibitor-based assay the combination of boronic acid with cefoxitin provided the best results, while the cefoxitin and boronic acid combination showed poor activity detecting 108 AmpC producers (Table 1). None of the ESBL-producing non-ampC (n=10) isolates showed inhibition with boronic acid. Production of KPC enzyme was not detected in any of the isolates, while co-production of ESBL along with AmpC was detected in 140 (30.8 %) isolates.

Thus, using cefotaxime as the antibiotic substrate clearly provided the best results, with 56.2 % prevalence compared to 64.6 % prevalence rate by M3DT. This might be due to the low level of AmpC production that was detected by M3DT but not the boronic acid-based test. The detection of AmpC activity by using either cefoxitin or cefotaxime lacked sensitivity with 23.7 and 47.4 % prevalence, respectively, compared to the 64.6 % prevalence rate by M3DT. The inhibition-based test yielded poor results with cefoxitin, which could be due to altered expression of outer-membrane proteins constituting porins, which can also contribute to cefoxitin resistance (Clarke et al., 2003).

On performing multiplex PCR, 103 isolates were confirmed positive and were found to be of five different families, of which the CIT family was the most predominant being harboured by 37 (35.9 %) isolates; in addition, DHA, ACC, EBC and FOX family were found in 12, 11, 5 and 10 isolates, respectively, while 28 isolates harboured multiple β-lactamase-encoding genes. The majority of the isolates showed negative results, which indicates they were most likely AmpC hyperproducers that showed positive results phenotypically because of overexpression of the chromosomal ampC gene, or they harboured a plasmid-encoded ampC gene that was not targeted by the primers used in the present study. On performing random amplification of polymorphic DNA analysis 42 different types of E. coli were found. Isolates expressing AmpC enzymes showed MIC50 and MIC90 values within the resistant range for cephalosporins and monobactam, while for carbapenems both MIC50 and MIC90 values were below the breakpoint.

The M3DT was considered as the gold standard in our study as all the isolates that tested positive by different methods were also confirmed as positive by M3DT. The best test sensitivity (87.1 %) was obtained with combination of ceftazidime along with boronic acid, and the specificity was found to be 100 %. The diagnostic accuracy of the combination was 88.99 %. The positive predictive value was 100 % and the negative predictive value was 57.1 %.

The results indicate that a ceftazidime plus boronic acid based assay may be promising as a stand alone test for the phenotypic detection of AmpC production in E. coli. Though further standardization of this method is required in respect to different geographical areas and different gene types, an approach to detection of AmpC status in E. coli using the combination of ceftazidime with an inhibitor, boronic acid, would be reliable, simple, readily available and helpful to formulation of a treatment strategy.

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**Table 1. Detection of AmpC β-lactamase by different phenotypic and genotypic methods and statistical analysis**

<table>
<thead>
<tr>
<th>Organism/test value</th>
<th>Cefoxitin disc test</th>
<th>M3DT</th>
<th>Boronic acid + cefoxitin</th>
<th>Boronic acid + cefotaxime</th>
<th>Boronic acid + ceftazidime</th>
<th>Multiplex PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> (n=455)</td>
<td>345 (75.8 %)</td>
<td>294 (64.6 %)</td>
<td>108 (23.7 %)</td>
<td>216 (47.5 %)</td>
<td>256 (56.3 %)</td>
<td>103 (22.6 %)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>–</td>
<td>–</td>
<td>0.367</td>
<td>0.735</td>
<td>0.871</td>
<td>–</td>
</tr>
<tr>
<td>Specificity</td>
<td>–</td>
<td>–</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>–</td>
</tr>
<tr>
<td>Positive predictive</td>
<td>–</td>
<td>–</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td>–</td>
</tr>
<tr>
<td>Negative predictive</td>
<td>–</td>
<td>–</td>
<td>0.215</td>
<td>0.395</td>
<td>0.573</td>
<td>–</td>
</tr>
<tr>
<td>Total diagnostic accuracy (%)</td>
<td>–</td>
<td>–</td>
<td>46.09</td>
<td>77.39</td>
<td>88.99</td>
<td>–</td>
</tr>
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