Detection of extended-spectrum β-lactamases in members of the family Enterobacteriaceae: comparison of the Combination Double Disc Test Method and the Etest ESBL.

Anushree Basu¹, Uma Arumugam², Narahari Rao³

¹(Assistant Professor, Department of Microbiology, Sri Sai College of Dental Surgery, NTRUHS, Vikarabad, Andhra Pradesh, India)
²(Associate Professor, Shrimp Disease Diagnosis Lab, Vaccine Research Centre-Viral Vaccine, TANUVAS, MMC, Chennai 600051, India)
³(Professor & Head, Department of Pharmacology, Sri Sai College of Dental Surgery, NTRUHS, Vikarabad, Andhra Pradesh, India)

Abstract: Purpose: To evaluate the performance of the Combination Double Disc Method in the identification of ESBL-producing Enterobacteriaceae isolates. Materials and Methods: A total of 44 clinically relevant Enterobacteriaceae isolates were examined. The ESBL classification furnished by Combination Double Disc Test Method was concordant with that of the comparison method (Etest ESBL and molecular identification of beta-lactamases genes) for 18 (41%) of the 44 isolates evaluated. Results: ESBL production was correctly detected in 41% ESBL-producing organisms with at least one of the combination disks (sensitivity, 80%). Conclusion: In our study, Combination Double Disc Test Method appears to be a rapid and reliable tool for routine identification of ESBL-producing isolates of Enterobacteriaceae.

Keywords: blaCTX-M-like gene, CTX-M-3 β-lactamases, Combination Double Disc Test Method, ESBLs, Multidrug-resistance.

I. Introduction

Extended-spectrum beta-lactamases (ESBLs) observed in Escherichia coli and Klebsiella spp are a large, rapidly evolving group of plasmid-mediated enzymes that confer resistance to the oxyimino cephalosporins and monobactams. They are inhibited by clavulanate (CA), sulbactam, or tazobactam [1].

Current disc diffusion and automated susceptibility test methods do not reliably detect ESBL production. All molecular methods such as polymerase chain reaction (PCR), PCR–restriction fragment length polymorphism and direct nucleotide sequencing, which demonstrate ESBL production, are of variable sensitivity and may be time consuming, expensive or technically difficult to perform [2]. There is a need for an easy, rapid and reproducible method for the detection of ESBLs, suitable for use in the routine diagnostic laboratory. Additionally, if the method could utilize the same methodology as antimicrobial sensitivity testing, the use of extra two or three discs only would enable all clinical isolates to be screened during routine sensitivity testing.

Combination Double Disc Test Method of ESBL detection, which satisfies these criteria, is compared with another diffusion method in current use.

II. Methods

Study design and data collection

During the study period, on an average, five clinical cultures per week were positive for ESBL-producing Enterobacteriaceae. No outbreaks of ESBL-producing bacteria were found among clinical cultures. No additional infection control precautions were used for patients with ESBL-producing bacteria on clinical culture.

Doctors and nurses obtained specimens for culture from all patients admitted to either the surgical or medical ICU at a specialty hospital in southern India during September - October, 2008. The study was carried out with the approval from the hospital management.

Bacterial isolates: Numerous bacteria were isolated from various body sites of 81 patients. There were 59 gram-negative isolates with Haemophilus influenzae, Pseudomonas aeruginosa, Acinetobacter followed by the members of the Enterobacteriaceae including Escherichia coli, Klebsiella pneumoniae (44 in total).

Of these, Enterobacterial isolates from only those samples that represented infection were considered. The distribution of the Enterobacteriaceae and their sources of isolation are shown in (Table 1).

We evaluated a set of 18 ESBL-positive and 26 ESBL-negative isolates.

The 18 ESBL-positive organisms included Enterobacteriaceae whose ESBLs had previously been identified genotypically. The clinical isolates consisted of E. coli (n = 9), K. pneumoniae (n = 9) strains.
Twenty six ESBL-negative Enterobacterial isolates, including challenging isolates with mechanisms anticipated to be different than bla_{CTX-M-3}-like gene producers [e.g. AmpC hyperproducers, inhibitor resistant (IRT) β-lactamases], were included as negative controls. These isolates consisted of E. coli (n = 14), K. pneumoniae (n = 12).

Microbiological Methods

The cultures were processed for ESBL-producing bacteria in real time as the specimens were collected. Lactose-fermenting colonies growing on the ceftazidime-containing MacConkey agar plates (2 mg/mL) [3] were identified as Escherichia coli or Klebsiella species and confirmed by using rapid ID 32 E identification system (mini API, bioMerieux, France) [1].

Molecular Detection: All isolates (E. coli, Klebsiella pneumoniae) were subjected to PCR amplification of bla_{CTX-M-3} like genes as described previously [4]. This profile was used as the comparison method for the assessment of the performance of the Combination Double Disk Method and Epsilometer test.

Combination Double Disc Test Method: All E. coli and Klebsiella pneumoniae underwent ESBL confirmatory testing by Combination Double Disc Test method [5] in which cefotaxime (30µg), ceftazidime (30µg) and cefepime (30µg) were used alone and in combination with clavulanate (10µg). The tests were set up and results were interpreted according to CLSI guidelines (HiMedia, India) [6] [Fig.2].

ESBL production is confirmed in E. coli, Klebsiella pneumoniae, if testing in the presence of CA increases the diameter of the inhibition zone for these drugs by at least 5 mm (compared with results obtained with the cephalosporin alone). K. pneumoniae ATCC 700603 was included as positive control and Escherichia coli ATCC 25922 was included as positive control strain.

Epsilometer test: To confirm the clavulanic acid inhibitable ESBLs, 46 strains were further tested by the gradient diffusion method (Etest ESBL) using strips with ceftazidime alone and associated with clavulanate (AB Biodisk, Solna, Sweden); findings were interpreted according to the manufacturer’s instructions [27] [Fig.3]. K. pneumoniae ATCC 700603 were included as positive control strain in all sessions.

Verification and analysis of Combination Double Disc Test Method: The sensitivity and specificity values for the Combination Double Disc Method were calculated against the results of the molecular comparison method described above. When discrepancies emerged, molecular detection, Combination Double Disc Method and Etest were repeated.

III. Statistical Methods

The sensitivity (%) was calculated as follows: (number of isolates showing a combination discs positive result) × 100/number of isolates with a positive genotypic identification of ESBL production. The specificity (%) was estimated as follows: (number of isolates showing a negative result using the combination discs) × 100/number of isolates that failed to produce ESBLs positivity in PCR.

Comparison of sensitivity and specificity of each method were performed using the two-way ANOVA using the Graph pad prism 5 statistical software (San Diego, California).

IV. Results

Molecular Testing

PCR diagnosis revealed the presence of bla_{CTX-M-3} like genes in (18/44) 41% test isolates [Fig.1]. The ESBL producers were E. coli (n=9), K. pneumoniae (n=9).

Phenotypic Typing

Combination Double Disc Test Method: All E. coli, Klebsiella pneumoniae isolates also tested for the CLSI criteria for confirmation of ESBL production (i.e., disk diffusion zone diameters increased by ≥5 mm around ceftazidime, cefotaxime and cefepime disks in the presence of CA).

Cac proved more sensitive than Cec discs (72 versus 67%; P<0.05) for detecting ESBL-positive strains, while both tests yielded equivalent specificities (92 versus 100%; P<0.001). The Cac discs were found to be particularly accurate for detecting in Enterobacterial isolates. On the other hand, Cac was slightly more sensitive than Cef for detecting ESBL-positive K. pneumoniae isolates (67% versus 56%; P<0.05). However, there was no statistically significant difference in the sensitivity of Cac and Cec discs in detecting ESBLs in E. coli (both showing 78%). Interestingly, the use of Cac disc positive results increased the sensitivity of ESBL detection up to 80% whereas the specificity was determined to be 97% [Table 2 and Table 3].

Etest ESBL: Three isolates were considered ESBL producers when clavulanate caused a ≥3 twofold-concentration decrease (ratio, ≥8) in the MIC of ceftazidime in combination with a ceftazidime MIC ≥1 μg/mL. Etest MICs of ceftazidime for E.coli (n=2) and K.pneumoniae (n=1) were 2 μg/mL, 6 μg/mL; 2 μg/mL respectively, and the decreases observed in the presence of CA amounted to less than 3 twofold dilutions (MIC...
Detection of extended-spectrum β-lactamases in members of the family Enterobacteriaceae:

...range, 0.064 to 0.25 µg/liter). Additionally, other three strains were considered ESBL positive if a phantom zone or a deformation of the ceftazidime could be observed, independent of the ratios or MICs [Table 4].

Approximately 28% of the 18 ESBL carriers were classified by the Etest as susceptible (defined by a MIC of TZ/TZL <8).

The outcome of the test was indeterminate (7/18=39%) when MICs were outside the test range of the test device. It included three E.coli and four K. pneumoniae isolates.

V. Figures And Tables

![Figure 1: Detection of blaCTX-M-like genes using PCR](image1)

![Figure 2: Combination Double Disk Test with the disks containing cephalosporin-clavulanate (A1: Cefotaxime+CA, B1: Ceftazidime+CA, C1: Cefipime+CA) is placed in proximity to the disk containing cephalosporin alone (A: Cefotaxime, B: Ceftazidime, C: Cefipime)](image2)

![Figure 3: Etest ESBL strip (AB Biodisk, Solna, Sweden). The zone of inhibition is read from two halves of the strip containing ceftazidime alone (TZ) on left or ceftazidime plus clavulanate (TZL) on right](image3)

<table>
<thead>
<tr>
<th>Species</th>
<th>BAL</th>
<th>Blood</th>
<th>Urine</th>
<th>Sputum</th>
<th>Liver Abscess</th>
<th>Endo-tracheal Aspirate</th>
<th>Tracheostomy</th>
<th>Throat Swab</th>
<th>Bile</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>0</td>
<td>04</td>
<td>17</td>
<td>01</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>02</td>
<td>02</td>
<td>07</td>
<td>02</td>
<td>01</td>
<td>05</td>
<td>01</td>
<td>01</td>
<td>0</td>
</tr>
</tbody>
</table>

BAL: Bronchial Alveolar Lavage
Detection of extended-spectrum β-lactamases in members of the family Enterobacteriaceae:

<table>
<thead>
<tr>
<th>Organisms (no. of strains)</th>
<th>Genotypic detection of bla&lt;sub&gt;CTX-M&lt;/sub&gt;</th>
<th>No/Sensitivities (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cec ≥5 mm</td>
<td>Cac ≥5 mm</td>
</tr>
<tr>
<td>All (44)</td>
<td></td>
<td>12/67</td>
<td>13/72</td>
</tr>
<tr>
<td>E. coli (23)</td>
<td></td>
<td>7/78</td>
<td>7/78</td>
</tr>
<tr>
<td>Kleb. pneumoniae (21)</td>
<td></td>
<td>5/56</td>
<td>6/67</td>
</tr>
</tbody>
</table>

Table 3: Specificities of Combination Double Discs Test Method for the detection of ESBL according to species

<table>
<thead>
<tr>
<th>Organisms (no. of strains)</th>
<th>Genotypic detection of bla&lt;sub&gt;CTX-M&lt;/sub&gt;</th>
<th>No/Sensitivities (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cec ≥5 mm</td>
<td>Cac ≥5 mm</td>
</tr>
<tr>
<td>All (44)</td>
<td></td>
<td>26/100</td>
<td>24/92</td>
</tr>
<tr>
<td>E. coli (23)</td>
<td></td>
<td>14/100</td>
<td>14/100</td>
</tr>
<tr>
<td>Kleb. pneumoniae (21)</td>
<td></td>
<td>12/100</td>
<td>10/83</td>
</tr>
</tbody>
</table>

Table 4: E-test for Clinical Isolates

<table>
<thead>
<tr>
<th>Organisms</th>
<th>No. of isolates</th>
<th>TZ/TZL&lt;sup&gt;a&lt;/sup&gt; ratio of the Etest</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>23</td>
<td>3 (24–63)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kleb. pneumoniae</td>
<td>21</td>
<td>3 (31–187)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>TZ, MIC of ceftazidime (mg/L); TZL, MIC of ceftazidime plus clavulanic acid (mg/L).
<sup>b</sup>Ratio values or ranges; *ND, not determined because TZ and TZL values were >32 and >8 mg/L, respectively.

VI. Discussion

This study adopted Combination Double Disk Test & E-test which are more sensitive and specific than traditional susceptibility tests to detect ESBLs in clinical isolates.

Combination Double Discs Method

The Combination Double Discs Test Method using at least one of the three combination discs ie, ceftazidime, cefotaxime cefepime gave concordant results in all 18 isolates tested positive by PCR.

The sensitivity was increased to 80% if the results obtained using all the agents were taken into consideration. From this data, it appears that the simultaneous use of all three cefotaxime, ceftazidime and cefepime discs is important as ESBLs can be missed if just one of these is used.

In line with previous reports, our study confirmed the higher sensitivity of ceftazidime/clavulanate discs (Cac) compared with cefotaxime/clavulanate discs (Cec) for the detection of ESBLs among E. coli and Klebsiella spp. Isolates [8, 9].

Since plasmid-mediated AmpC enzymes have also been shown to disseminate among Enterobacteriaceae, sometimes in combination with ESBLs, cefepime (discs) which is relatively unaffected by high-level AmpC expression, were included in this study in order to obtain better sensitivity of the disc diffusion test [10]. Nevertheless, in our experience, cefepime/clavulanic acid discs (Cfc) with a zone size diameter cut-off value of at least 5mm indeed proved clearly superior to ceftazidime/clavulanic acid discs (Cec) &
Detection of extended-spectrum β-lactamases in members of the family Enterobacteriaceae:

cefotaxime/clavulanic acid (Cac). Further evaluation should be performed on a larger range of isolates from different origins to confirm such observations.

Furthermore, in one (K. pneumoniae) of the twenty six $bla_{CTX-M,3}$ negative isolates, CaC showed the highest zone size differences compared with the two other combination discs, which possibly suggests the occurrence of non CTX-M ESBLs such as TEM- or SHV-type ESBLs.

Based on our results, the use of both CaC and CeC discs appears to be the preferred combination for the detection of ESBL, irrespective of the bacterial species involved, Cfc being the most sensitive one.

**E-test**

In this study, Etest ESBL with ceftazidime detected only 6 out of 18 isolates of ESBLs tested (33%), whereas a report by Vercauteren et al. (1997) showed that the Etest ESBL test with ceftazidime could detect 81% of ESBLs tested in their laboratory [7].

Seven isolates with an indeterminate ESBL-Etest result, contained $bla_{CTX-M,3}$-like genotype. This phenomenon may suggest the presence of an inhibitor-resistant TEM or AmpC enzymes [2].

The strip method is convenient and easy to use. Though, the deformation of the inhibition ellipse has been added as a single criterion for the presence of an ESBL. But it is sometimes difficult to read the test when the MICs of ceftazidime are low because the clavulanate sometimes diffuses over to the side that contains ceftazidime alone [2].

**Comparison of Combined double disc diffusion test with Etest**

The Combined double disc diffusion test was then compared with the commercially available Etest. All tests were prepared by one individual and read independently, checked by the responsible laboratory staffs who were blinded to the identity of each strain.

There was significant difference in the accuracy of Comparison of Combined double disc diffusion test & Etest with ceftazidime. The Combined double disc diffusion test tends to have a higher sensitivity (80% versus 33%) with five ESBL-positive isolates that were missed by Etest. This is in coincidence with the results of Vercauteren et al. (1997) but contraindicates the findings of Johann et al. (2005) where Etest was shown to be more sensitive than the disk approximation test in detecting ESBLs in clinical isolates.

There was an excellent concordant with seven non-determinable (ND) results by ESBL E-test & combined double disc test using Cec & Cac discs. It is plausible that the presence of an ESBL may be masked by the concurrent expression of an AmpC-type enzyme in the same strain, because no inhibitory effect of clavulanic acid can be detected due to the clavulanate-insensitive AmpC-type enzyme.[2,8] While it was clearly evidenced that Cfc could efficiently detect ESBLs not affected by AmpC-type enzyme.

Although Etest has been proposed as a simple technique for the detection of ESBL production [11], it is comparatively expensive, therefore cannot easily be incorporated into a routine disc testing system. In opposite, combined double disc test requires only three discs of cephalosporins alone and in combination with clavulanic acid to be added to the sensitivity plate, enabling all Gram-negative bacteria to be screened in the diagnostic laboratory. Moreover, this particular method poses as an alternative to other time consuming, confusing, complicated & expensive disk diffusion methods (e.g. three dimensional disk tests) generally practiced in most of the laboratories [12].

**VII. Conclusions**

This study finds Combined Double Disc Test to be the reliable phenotypic method for ESBLs detection. In addition, the selection of both Cec and Cac discs appears to be the optimal combination for ESBL detection among various Enterobacteriaceae isolates, since it significantly increased the sensitivity of the tests. Moreover, our results, based on the detection of $bla_{CTX-M,3}$-like genes by PCR, suggest that Cfc discs at a ≥5 mm threshold value may constitute an accurate alternative to Cec & Cac discs for the detection of ESBL enzymes among E.coli & K.pneumoniae.

Likewise, it is deemed very important to develop and evaluate routinely applicable laboratory techniques that are the most suited for the detection of ESBLs.

Finally, as ESBLs are subject to rapid evolution and are involved in a dynamic epidemiology, it should be noted that the results of this study or any other study addressing the same question might not be applicable to every laboratory worldwide at any moment in time.

**Acknowledgement**

We gratefully acknowledge Apollo Speciality Hospital, Chennai, India for approving and providing facilities to carry out this research.
Detection of extended-spectrum β-lactamases in members of the family Enterobacteriaceae:

References


