Comparison of Disc Diffusion Methods for the Detection of Extended-Spectrum Beta Lactamase-Producing Enterobacteriaceae

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ABSTRACT

Background: Resistance to broad-spectrum β-lactams, mediated by extended-spectrum β-lactamases (ESBLs), is an increasing problem worldwide. This resistance poses problems for in vitro testing and reporting. Increased prevalence of ESBLs among Enterobacteriaceae creates a great need for laboratory testing methods that will accurately identify their presence.

Materials and Methods: During the study, the Enterobacteriaceae isolated were tested for the presence of ESBL by the National Committee for Clinical Laboratory Standards (NCCLS) screening test, Jarlier double disc synergy (approximation) test (DDST) and NCCLS phenotypic confirmatory test (PCT), and compared their efficiency in detection.

Results: A total of 313 Enterobacteriaceae were isolated and tested for the presence of ESBL. NCCLS PCT identified 200 (63.89%) as ESBL producers and DDST identified 176 (56.23%), with a P-value of <0.001. Among the screening agents, ceftazidime had a better sensitivity (89.49%) and specificity (95.74%).

Conclusions: Close monitoring of the susceptibility pattern of isolates and careful spacing with specific discs can identify many ESBL producers. Ceftazidime has a better sensitivity and specificity as a screening agent. A combination of different tests can be useful for accurate identification.

Keywords: DDST, Enterobacteriaceae, ESBLs, NCCLS screening, PCT
A significant proportion of ESBL-producing organisms may have elevated mean inhibitory concentrations (MICs) to ceftazidime that do not reach the National Committee for Clinical Laboratory Standards (NCCLS) break point for resistance (MIC >8 µg/ml) and, therefore, may be incorrectly dismissed as non-ESBL producing organisms.\(^7\)

The increased prevalence of Enterobacteriaceae producing ESBLs creates a great need for laboratory testing methods that will accurately identify the presence of these enzymes in clinical isolates.\(^6\) Hence, this study was undertaken to detect ESBL producers by using NCCLS screening test, Jarlier double disc synergy (approximation) test (DDST) and NCCLS phenotypic confirmatory test (PCT), and to compare their efficiency in detection.

**MATERIALS AND METHODS**

A total of 313 consecutive non-repeat culture isolates of Enterobacteriaceae were obtained from 280 different specimens from different specialty wards. The isolates were identified on the basis of conventional microbiological procedures like their growth pattern on blood agar and Mac Conkey’s agar. The characters assessed included morphology on Gram’s staining, motility, methyl red test, Vogues-Proskauer test, citrate utilization, catalase, indole and urease production, nitrate reduction, sugar fermentation and amino acid decarboxylation and arginine dihydrolase test. Antimicrobial susceptibility testing was determined by the Kirby-Bauer disc diffusion method as per the NCCLS recommendations.\(^8\)

**NCCLS screening test**

Isolates showing an inhibition zone size of ≤22 mm with ceftazidime (30 µg), ≤25 mm with ceftriaxone (30 µg) and ≤27 mm with cefotaxime (30 µg) were identified as potential ESBL producers and were short listed for confirmation of ESBL production.

**Double disc approximation test/DDST**

First, using the detection test described by Jarlier et al.\(^{6,9}\) synergy was determined between a disc of amoxicillin–clavulanic acid (20 µg/10 µg) (augmentin) and a 30-µg disc of each third-generation cephalosporin test antibiotic placed at a distance of 20 mm from center to center on a Mueller-Hinton Agar (MHA) plate swabbed with the test isolate. Clear extension of the edge of the inhibition zone of cephalosporin toward the augmentin disc was interpreted as positive for ESBL production.\(^{6,9-11}\)

**NCCLS phenotypic confirmatory combination disc diffusion test**

A disc of ceftazidime (30 µg) alone and ceftazidime + clavulanic acid (30 µg/10 µg) were placed at a distance of 25 mm, center to center, on a MHA plate inoculated with a bacterial suspension of 0.5 McFarland turbidity standards and incubated overnight at 37°C.

An increase in the inhibition zone diameter of ≥25 mm for a combination disc versus ceftazidime disc alone confirmed ESBL production.\(^7,8\)

The Kirby Bauer plates for the susceptibility tests were prepared in the laboratory. All of the antibiotic discs and dehydrated media were obtained from Hi-Media Laboratories Pvt. Limited, Mumbai, India.

**Statistical analysis**

The chi-square test was used with appropriate correction for the observation using EPI 6 software.

**RESULTS**

Among the 313 Enterobacteriaceae isolates, Klebsiella sp. was the most common pathogen isolated from the tested samples, constituting 115/313 (36.74%) of the total isolates, followed by Escherichia coli 82/313 (26.17%), Providencia sp. 25/313 (7.98%), Proteus vulgaris 24/313 (7.66%) and Proteus mirabilis 18/313 (5.75%). All the 313 Enterobacteriaceae isolates were subjected to the initial NCCLS screening test, followed by the DDST and the NCCLS PCT. The PCT identified 200/313 (63.89%) as ESBL producers, whereas the DDST at a distance of 20 mm between center to center identified only 176/313 (56.23%), with a P-value of <0.001, which was highly significant. The DDST had a sensitivity of 94.89% (167/176, 95% confidence interval [CI] 91.57–98.21), a specificity of 75.91% (104/137, 95% CI 72.26–79.56) and a positive predictive value of 83.55% (167/200) and negative predictive value of 92.03% (104/113).

Among the third-generation cephalosporins used for screening, ceftazidime had a better sensitivity and specificity, followed by ceftriaxone. Even though cefotaxime had a good specificity, it lacked sensitivity [Table 1]. During the screening procedure, ceftazidime, cefotaxime and ceftriaxone identified 219, 248 and 226 isolates, respectively, as potential ESBL producers.

The distribution of ESBL producers varied among
different species of *Enterobacteriaceae*. The rates were high among *Klebsiella oxytoca* (89.47%), *Klebsiella pneumoniae* (71.87%), *Escherichia coli* (62.19%), *Proteus mirabilis* (61.11%), *Proteus vulgaris* (50.00%) and Providencia sp. (44.00%), which were the major isolates in the group.

## DISCUSSION

ESBLs are a problem in hospitalized patients throughout the world. The prevalence of ESBL among clinical isolates varies greatly worldwide and within geographic areas, and is rapidly changing over time. This increased prevalence of *Enterobacteriaceae* producing ESBLs creates a great need for laboratory testing methods that will accurately identify the presence of these enzymes in clinical isolates.

The various susceptibility testing methods differ in their ability to detect cephalosporin resistance in the ESBL-producing strains.

In a study by Singhal *et al.*, four different tertiary care hospitals from across India identified a prevalence rate of 63.60% by NCCLS methods and Mathur *et al.*, from AIIMS, New Delhi, reported a prevalence rate of 68% for ESBL production among Gram negative bacilli by the NCCLS confirmatory tests. The present study, with a prevalence rate of 63.89% by NCCLS PCT, correlates well with these studies.

ESBL-producing bacteria may appear falsely susceptible to certain extended-spectrum cephalosporins in *in vitro* susceptibility testing when National guidelines are used. A second test has therefore been recommended for the detection of ESBL activity. The DDST is the most widely used test due to its simplicity and ease of interpretation. It is a reliable method for the detection of ESBLs. However, the sensitivity of the DDST in different studies ranges from 79% to 96%. This lack of sensitivity results from the fact that DDST is not a standardized procedure.

The sensitivity of DDST varies with the distance between the discs. Ho *et al.* reported the sensitivity of DDST to be 83.8% at a single interdisc width of 30 mm compared with the inhibitor-potentiated disc diffusion test. They also reported that the sensitivity can be increased to 97.9% by decreasing the interdisc width to 20 mm. Vercauteren *et al.* reported sensitivities of the double disc and three-dimensional tests at 96.9% and 90.6%, respectively. The DDST sensitivity in the present study correlates well with those studies.

Zali *et al.* reported that the clinical strains producing SHV–6 ESBL and Amp C type β-lactamase producers would not be detected by double disc diffusion tests or the MAST (MAST Laboratories Ltd., Bootle, Merseyside, UK) double disc test (MDD). They also noted that Amp C type β-lactamase producers give negative results with the double disc, MDD and Epsiloemeter (E) test methodologies.

The commonly used disc diffusion method was insufficient for the detection of ESBL activity using ceftazidime alone as an indicator. In contrast, reduced susceptibility to aztreonam and other cephalosporins resulted in an acceptable ESBL detection rate. This reflects the common occurrence of ESBL with a low specificity for ceftazidime. Recent data suggest that susceptibility testing with cefpodoxime can lead to a high number of false-positives if the current NCCLS interpretative criteria are applied.

While studying ESBLs using the NCCLS criteria for screening tests, Ho *et al.* reported sensitivities of 57.7% for cefazidime, 98.6% for ceftazidime, 99.3% for cefpodoxime and 93% for Aztreonam using the Kirby Baeur disc diffusion method.

However, Vercauteren *et al.* detected only 48% of the ESBL-producing reference strains by their reduced susceptibility to ceftazidime and by combining ceftazidime, cefazidime and aztreonam, they detected only 52% of the ESBL-producing strains. In the present study, ceftazidime had a better sensitivity (89.49%) and specificity (95.74%) compared with ceftazidime (79.83% and 92.30%) as a screening agent. Even though cefotaxime has a good specificity (95.4%), it lacks sensitivity (86.72%).

Although molecular methods and automated systems appear to be sensitive in ESBL detection, they are expensive, time consuming and require specialized equipment and expertise. Commercially available E tests have been proposed as simple techniques for the detection of ESBL production, but they are costly and need extra MHA plates. In places where resources are minimal and workloads are high, close monitoring of susceptibility...
patterns of members of *Enterobacteriaceae* isolates by screening methods such as DDST and NCCLS PCT can detect many ESBL producers.\[19\] However, careful spacing and use of specific discs are required for accurate results. The use of these tests may contribute to wider recognition and more scrupulous monitoring for the presence of emerging drug-resistant organisms.

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