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EVALUATION OF A MODIFIED DOUBLE-DISC SYNERGY TEST FOR DETECTION OF EXTENDED SPECTRUM β-LACTAMASE-PRODUCING PROTEUS MIRABILIS

MKR Khan, *SS Thukral, R Gaind

Abstract

The detection of extended-spectrum β-lactamases (ESBLs) in gram-negative bacteria that produce AmpC β-lactamases is problematic. In the present study, the performance of modified double-disc synergy test (MDDST) that employs a combination of cefepime and piperacillin-tazobactam for the detection of Proteus mirabilis producing extended spectrum and AmpC β-lactamases was evaluated and compared with double-disc synergy test (DDST) and NCCLS phenotypic disc confirmatory test (NCCLS-PDCT). A total of 90 clinical isolates of P. mirabilis, which met the CLSI (Clinical and Laboratory Standards Institute) screening criteria that these had broth microdilution (BMD) MIC of ≥2 µg/mL for at least one extended spectrum cephalosporin [ceftazidime (CAZ), cefotaxime (CTX) and cefpodoxime], were selected for the study. MDDST detected ESBLs in 40/90 of the isolates, whereas DDST detected ESBLs in only 25 isolates. NCCLS-PDCT could detect ESBLs in 39 isolates using CAZ and CAZ + clavulanic acid (CLA) combination, whereas CTX and CTX + CLA combination could detect only 37 isolates as ESBL positive. As many as 34/40 ESBL positive isolates were confirmed to be AmpC β-lactamase positive by the modified three-dimensional test (MTDT). MDDST and NCCLS-PDCT could detect ESBLs in all the 34 AmpC positive isolates, whereas DDST could detect ESBLs in only 19 isolates. The study demonstrated that MDDST is superior to DDST and as sensitive as NCCLS-PDCT. However, MDDST seems to have enhanced potential for the detection of ESBLs in AmpC β-lactamase-producing P. mirabilis.

Key words: AmpC β-lactamase, extended-spectrum β-lactamase detection, Proteus mirabilis

Extended-spectrum β-lactamases (ESBLs) are mutant, plasmid-mediated β-lactamases derived from older, broad-spectrum β-lactamases and confer resistance to all extended-spectrum cephalosporins (ESCs) and aztreonam, except cephamycins and carbapenems,[1,2] ESBLs, although most commonly encountered in Klebsiella spp. and Escherichia coli, have also been detected in other gram-negative bacteria, including Enterobacter, Salmonella, Citrobacter, Serratia marcescens, Proteus spp. and Pseudomonas aeruginosa.[2,3] AmpC β-lactamases are cephalosporinases that are poorly inhibited by clavulanic acid (CLA) and can be differentiated from ESBLs by their ability to hydrolyse cephemycins.[4] A wide variety of bacterial species, viz. E. coli, Klebsiella pneumoniae, Proteus mirabilis, Enterobacter aerogenes, Salmonella spp. and Citrobacter freundii, have been shown to harbour AmpC β-lactamases.[5,6] The detection of ESBLs in AmpC-producing species of gram-negative bacteria is problematic. The increased prevalence of bacterial pathogens producing both ESCs and AmpC β-lactamases creates a requirement for laboratory testing methods that can accurately detect the presence of these enzymes in clinical isolates.[7] The inhibitor-based confirmatory test approach is most promising for isolates that do not co-produce an inhibitor-resistant β-lactamase like AmpC. However, a high-level production of AmpC may prevent the detection of an ESBL. This problem is frequently observed in tests with species or strains that produce a chromosomally encoded inducible AmpC β-lactamase (e.g., Enterobacter spp., Citrobacter spp., Serratia spp., Proteus spp. and Pseudomonas aeruginosa). Moreover, in these organisms, CLA may act as an inducer of high-level AmpC production resulting in an increase in the resistance of the isolate to other screening drugs, producing a false-negative result in the ESBL detection test. Tazobactam and sulbactam are much less likely to induce AmpC β-lactamases and are, therefore, preferable inhibitors for ESBL detection tests with these organisms.[8] Another approach is to include cefepime (FEP) as an indicator drug.[9] High-level AmpC production has a minimal effect on the activity of FEP, making this drug a more reliable detection agent for ESBLs in the presence of an AmpC β-lactamase.[1] A test incorporating FEP and piperacillin-tazobactam (TZP) for the detection of Enterobacteriaceae that produce extended spectrum and AmpC β-lactamases has been described.[7] We describe here a modified double-disk synergy test (MDDST), which differs from the original double-disc synergy test[9] in two respects; first, addition of discs of FEP and TZP; second, adjustment of the distances between various discs for accurately detecting the synergy between augmentin/TZP and extended-spectrum cephalosporin. The test was evaluated for detecting

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ESBL in AmpC β-lactamase-producing *P. mirabilis* and compared with original double-disc synergy test (DDST) and NCCLS phenotypic disc confirmatory test (NCCLS-PDCT). The production of AmpC β-lactamases in ESBL positive isolates was confirmed by the modified three-dimensional test (MTDT).

**Materials and Methods**

A total of 90 consecutive, non-repetitive clinical isolates of *P. mirabilis* recovered over a period of nine months (August 2004 to April 2005) from a variety of clinical specimens, viz., urine, pus, wound swab and high vaginal swab, processed at Safdarjung Hospital and Associated Vardhman Mahavir Medical College, New Delhi, and Maulana Azad Medical College and Associated Chacha Nehru Children Hospital, New Delhi, were studied.

**Screening for ESBLs**

The test was put up in 96-well microtitre plates using cation-adjusted Mueller-Hinton broth. Isolates having MICs of ≥2 µg/mL for ceftazidime (CAZ) or cefotaxime (CTX) as per the NCCLS/CLSI (Clinical and Laboratory Standards Institute) screening criteria were taken up for further study.[10]

**Detection of ESBLs**

### Double-disc synergy test

The test inoculum (0.5 McFarland turbidity) was spread onto Mueller-Hinton agar (MHA; HiMedia, India) using a sterile cotton swab. A disc of augmentin (20 µg amoxyxillin + 10 µg CLA) was placed on the surface of MHA; then discs of cefpodoxime (30 µg), CAZ (30 µg) and CTX (30 µg) were kept around it in such a way that each disc was at distance ranging between 16 and 20 mm from the augmentin disc (centre to centre). The plate was incubated at 37 °C overnight. Distances between the discs were required to be suitably adjusted for each strain in order to accurately detect the synergy. The organisms were considered to be producing ESBL when the zone of inhibition around FEP or any of the extended-spectrum cephalosporin discs showed a clear-cut increase towards the TZP disc. The discs of ciprofloxacin (5 µg), amikacin (30 µg), gentamicin (10 µg) and cefoxitin (30 µg) were also included so as to find out the susceptibility of the isolates to commonly used antibiotics (Fig. 1).

### Phenotypic disc confirmatory test

This test was performed as the disc diffusion test as recommended by CLSI (formerly NCCLS).[10] A ≥5 mm increase in zone diameter for either CAZ or CTX tested in combination with CLA, versus its zone diameter when tested alone, confirmed an ESBL-producing organism. *E. coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC 700603) were used as negative and positive control reference strains, respectively.

**Detection of AmpC β-lactamases**

### Modified three-dimensional test

The presence of AmpC β-lactamases in ESBL positive isolates with reduced susceptibility to cefoxitin was detected by MTDT.[4] Briefly, fresh overnight growth from MHA was transferred to a preweighed sterile microcentrifuge tube. The tube was weighed again to determine the weight of bacterial mass to obtain 10-15 mg of bacterial wet weight. The bacterial mass was suspended in peptone water and pelletted by centrifugation at 3000 rpm for 15 minutes. Crude enzyme extract was prepared by freezing and thawing the bacterial pellet (five cycles). Lawn culture of *E. coli* ATCC 25922 was prepared on MHA plates, and a cefoxitin (30 µg) disc was placed on the surface of the medium. Linear slits (3 cm long) were cut using sterile surgical blade up to a point 3 mm away from the edge of the cefoxitin disc. Wells of 8 mm diameter were made on the slits at a distance 5 mm inside from the outer end of the slit using a sterile Pasteur pipette. The wells were loaded with enzyme extract in 10 µL increments until the well was full. Approximately 30-40 µL of extract was loaded in a well. The plates were incubated at 37 °C overnight. Three different kinds of results were recorded. Isolates that showed clear distortion of zone of inhibition of cefoxitin were taken as AmpC producers. Isolates with no distortion were taken as AmpC non-producers, and isolates with minimal distortion were taken as intermediate strains. A known AmpC-positive isolate of *Klebsiella pneumoniae* was used as control reference strain (Fig. 2).

**Results**

Of the 90 isolates tested, MDDST detected ESBLs in 40 isolates, and DDST could detect in only 25. In DDST, CTX was found to be the best substrate, as it revealed synergism with augmentin in 24 out of 25 isolates. On the other hand, FEP and CAZ showed synergism in only two
In our study, MDDST, consisting of a combination of FEP and TZP, was shown to be the most sensitive test for ESBL detection in AmpC-positive *P. mirabilis*. MDDST detected the maximum number of ESBL-producing strains, followed by NCCLS-PDCT, whereas DDST could detect the least number of ESBL-positive isolates. Interestingly, MDDST could detect ESBL in one isolate of *P. mirabilis* that showed a negative result with both DDST and NCCLS-PDCT. The sensitivity of MDDST with respect to *P. mirabilis*, as observed in our study, is similar to that reported for isolates of *Enterobacteriaceae* other than *P. mirabilis* in an earlier study.[7] MDDST and NCCLS-PDCT, which detected ESBLs in 34/34 (100%) of the AmpC-producing isolates, were more sensitive than DDST, which could detect ESBL in only 25/34 (~63%) isolates. Similarly, DDST was found less sensitive than NCCLS-PDCT since it could detect ESBLs in 25 of the 39 isolates that were confirmed ESBL positive by the later technique. An earlier study using *Klebsiella pneumoniae* isolates had reported NCCLS-PDCT to be more sensitive than DDST.[11] AmpC β-lactamases are resistant to β-lactamase inhibitors like CLA. The detection of ESBLs in strains that produce inducible chromosomal AmpC β-lactamase is nearly impossible with confirmatory tests using clavulanate as the ESBL inhibitor, since AmpC β-lactamases do interfere with the inhibition of ESBLs by clavulanate.[1] False-negative results using DDST or NCCLS-PDCT have been reported in some earlier studies.[7,12] A negative ESBL confirmatory test using clavulanate as the inhibitor may be interpreted as an indication of AmpC production or reduced outer membrane permeability. A positive three-dimensional test result with cefoxitin demonstrates hydrolysis of cefoxitin and differentiates between AmpC production and reduced outer membrane permeability.[8] MDDST and NCCLS-PDCT could detect ESBLs in all the 34 AmpC-positive isolates. In our study, a high degree of co-resistance to cefoxitin (98%), gentamicin (~96%), amikacin (~93%) and ciprofloxacin (~82%) was observed in the ESBL-positive isolates. This is in contrast to an earlier study carried out in China where all the ESBL-producing isolates of *P. mirabilis* were found to be sensitive to cefoxitin and amikacin, whereas 38.5 and 76.9% of the isolates were resistant to gentamicin and ciprofloxacin, respectively.[3] All our ESBL/AmpC-positive isolates were sensitive to imipenem and TZP. An earlier study showed TZP as well as amikacin and meropenem to be important therapeutic options for infections due to multidrug-resistant, ESBL-producing *P. mirabilis*.[13]

In conclusion, the present practice to confirm ESBL production by carrying out tests, viz., DDST and NCCLS-PDCT, using clavulanate as the ESBL inhibitor with CAZ, CTX or cefpodoxime may not be useful in *P. mirabilis*, since a large percentage of these also produce AmpC β-lactamases. MDDST, on the other hand, is more sensitive and hence a better alternative. To the best of our knowledge, this constitutes the first report in which MDDST has been evaluated for the detection of ESBLs in AmpC-producing *P. mirabilis*.

**Discussion**

In our study, MDDST, consisting of a combination of FEP and TZP, was shown to be the most sensitive test for ESBL detection in AmpC-positive *P. mirabilis*. MDDST detected the maximum number of ESBL-producing strains, followed by NCCLS-PDCT, whereas DDST could detect the least number of ESBL-positive isolates. Interestingly, MDDST could detect ESBL in one isolate of *P. mirabilis* that showed a negative result with both DDST and NCCLS-PDCT. The sensitivity of MDDST with respect to *P. mirabilis*, as observed in our study, is similar to that reported for isolates of *Enterobacteriaceae* other than *P. mirabilis* in an earlier study.[7] MDDST and NCCLS-PDCT, which detected ESBLs in 34/34 (100%) of the AmpC-producing isolates, were more sensitive than DDST, which could detect ESBL in only 25/34 (~63%) isolates. Similarly, DDST was found less sensitive than NCCLS-PDCT since it could detect ESBLs in 25 of the 39 isolates that were confirmed ESBL positive by the later technique. An earlier study using *Klebsiella pneumoniae* isolates had reported NCCLS-PDCT to be more sensitive than DDST.[11] AmpC β-lactamases are resistant to β-lactamase inhibitors like CLA. The detection of ESBLs in strains that produce inducible chromosomal AmpC β-lactamase is nearly impossible with confirmatory tests using clavulanate as the ESBL inhibitor, since AmpC β-lactamases do interfere with the inhibition of ESBLs by clavulanate.[1] False-negative results using DDST or NCCLS-PDCT have been reported in some earlier studies.[7,12] A negative ESBL confirmatory test using clavulanate as the inhibitor may be interpreted as an indication of AmpC production or reduced outer membrane permeability. A positive three-dimensional test result with cefoxitin demonstrates hydrolysis of cefoxitin and differentiates between AmpC production and reduced outer membrane permeability.[8] MDDST and NCCLS-PDCT could detect ESBLs in all the 34 AmpC-positive isolates.

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**Figure 2:** Modified three-dimensional test showing AmpC positive (A) and negative (B) results
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