MODIFIED DOUBLE-DISC SYNERGY TEST (MDDST) FOR DETECTION OF EXTENDED-SPECTRUM β-LACTAMASES IN AmpC β-LACTAMASE-PRODUCING *KLEBSIELLA* CLINICAL ISOLATES

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Keywords: AmpC β-lactamase, extended-spectrum β-lactamases (ESBLs), *Klebsiella spp*., modified double-disc synergy test (MDDST), double-disc synergy test (DDST), phenotypic confirmatory test (E-test), modified three-dimensional test (MTDT)

The detection of extended spectrum β-lactamases ($ESBL_{(S)}$) 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 $ESBL_{(S)}$ *Klebsiella* producing AmpC β-lactamases was evaluated and compared with double-disc synergy test (DDST). E-test phenotypic confirmatory and modified three-dimensional tests (MTDT) were adopted for more data confidence. A total of 100 clinical isolates of *Klebsiella*, which met the CLSI (2012) screening criteria as having broth microdilution (BMD) MIC > or =2 mg mL^{-1} for at least one extended-spectrum cephalosporin [ceftazidime (CAZ), cefotaxime (CTX) and cefpodoxime], were accurately-selected for the study. MDDST detected ESBLs in 62 out of the100 studied isolates with 100 % sensitivity and specificity, whereas DDST detected ESBLs in only 52 isolates with 92.9 % sensitivity and 100 % specificity. E-test could detect ESBLs in 62 isolates, while as many as 34/62 ESBL positive isolates were confirmed to be AmpC beta-lactamase positive by the MTDT. MDDST and E-Test could detect ESBLs in all the 34 AmpC positive isolates, whereas DDST detects ESBLs in only 26 isolates. The study recommended MDDST as superior to DDST for the detection of ESBLs in AmpC β-lactamase-producing *Klebsiella spp*. and this was confirmed by MTDT and E-Tests.

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Introduction

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 cephamycin and carbapenem derivatives.[1,2] Although ESBLs have been detected in Gram-negative bacteria such *Enterobacter*, *Salmonella*, *Citrobacter*, *Serratia marcescens*, *Proteus* spp. and *Pseudomonas aeruginosa*,[2,3] it is most commonly encountered in K*lebsiella spp.* AmpC β-lactamases are cephalosporinases that are poorly inhibited by clavulanic acid (CLA) and can be differentiated from ESBLs by their ability to hydrolyze cephamycins.[4] A wide variety of Gram-negative bacteria, *Klebsiella pneumonia*, in particular, have been shown to harbor 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 ESBLs 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., *Klebsiella* spp., *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.[1] 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 an extended spectrum and AmpC β-lactamases have been described.[7]

We describe here a modified double-disc synergy test (MDDST), which differs from the original double-disc synergy test[9] in two respects; first, the 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 ESBL in AmpC β-lactamase-producing *Klebsiella* and compared with original double-disc synergy test (DDST) and phenotypic confirmatory test (E-test). The production of AmpC β-lactamases in ESBL positive isolates was confirmed by the modified three-dimensional test (MTDT).

Material and Methods

A total of 100 consecutive, non-repetitive clinical isolates of *Klebsiella* isolates was collected from a variety of clinical specimens, viz., urine, pus, wound swab and high vaginal swab, referred to the Central Microbiology Laboratory of Ain Shams University Hospitals for routine culture and sensitivity from September to December 2015.

Screening for ESBLs -Disc diffusion method (DDM)

The test inoculum (0.5 McFarland turbidity) was spread onto Mueller-Hinton agar plate, an interpretation of zone diameter of (CPD ≤17mm, CAZ ≤ 22 and CTX ≤27), this may indicate ESBL production (i.e.) positive screening for ESBL. However, FEP disc not included by CLSI (Clinical and Laboratory Standards Institute) for ESBL screening but FEP zone diameter FEP≤14mm indicate resistance which we considered as presumptive ESBL.[10]

Detection of ESBLs-Double-disc synergy test

The test inoculum (0.5 McFarland turbidity) was spread onto Mueller-Hinton agar (MHA; HiMedia) using a sterile cotton swab. A disc of augmentin (20 µg Amoxycillin + 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 any of the expanded-spectrum cephalosporin discs showed a clear-cut increase towards the Augmentin disc.[9]

Modified double-disc synergy test

The original DDST was modified for detecting ESBLs in AmpC-producing clinical isolates of *P. mirabilis* by placing a disc of TZP (100/10 µg) at a distance ranging between 22 and 25 mm from FEP (30 µg) disc. Briefly, a disc of augmentin (20 µg amoxycillin + 10 µg CLA) was placed on the surface of MHA; then discs of cefpodoxime (30 µg), CAZ (30 µg), CTX (30 µg) and FEP (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), and a disc of TZP (100/10 µg) was placed at a distance ranging between 22 and 25 mm from the FEP disc. Distances between the discs were required to be suitably adjusted depending on the zone of inhibition obtained with extended-spectrum cephalosporin disc in a particular isolate 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 Figure 1.

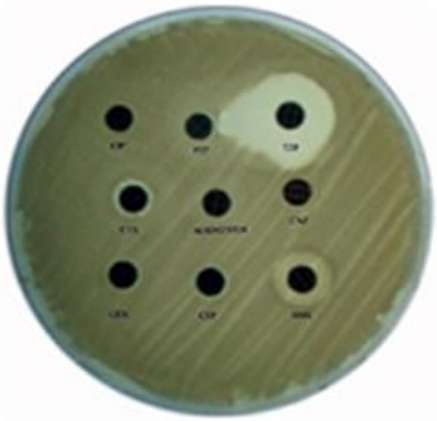

Figure 1. Modified double-disc synergy test (MDDST) showing synergy between cefepime (FEP)/ceftazidime (CAZ) and piperacillin-tazobactam (TZP)

Phenotypic disc confirmatory test - E-test

This test was recommended by CLSI (E-test ESBL strip).[10] The MIC value was read from the scale in terms of ug/ml where the ellipse edge intersects the strip. ESBL production is inferred if the MIC ratio for cephalosporin alone/cephalosporin plus clavulanate MIC is ≥8. ESBL production was also identified by the presence of a phantom zone or a deformation of the CAZ inhibition zone independent of the MIC ratios. If the MIC ratio is <8 it is indicative of non-ESBL production. When MIC values were above the test device range, the interpretation was 'non-determinable'.

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 reweighed 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 pelleted 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 the 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 (Figure 2).

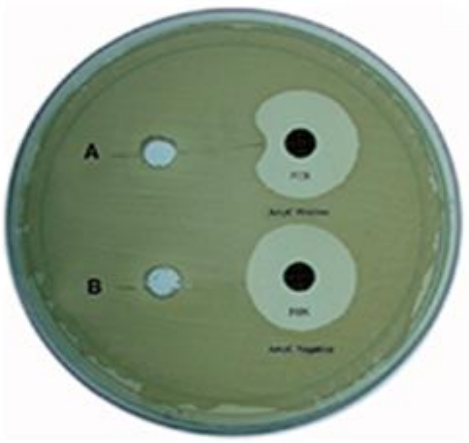

Figure 2. Modified three-dimensional test showing AmpC positive (A) and negative (B) results

Results

Sixty two/100 isolates were ESBL positive by E-test (golden standard test). Fifty-two out of 62 were positive by DDST, while MDDST detected all the 62 ESBL positive isolates. Sixty-seven out of 100 isolates were AmpC producers by M3D (golden standard test). Sixty-four out of 67 isolates that were positive by M3D test were resistant to FOX with a sensitivity of 95.5 % and specificity of 63.6 % for detection of AmpC production with positive predictive value and negative predictive value of (84.2% and 87.5 %) respectively. Sixty-seven out of 100 isolates were positive AmpC by M3D test. Thirty-four out of 67 isolates were positive ESBL by MDDST. On the other hand, 26/67 were positive by DDST. The sensitivity of DDST was 76.5 % and specificity of 100 % for detection of ESBL in presence of AmpC. Thirty-three out of 100 isolates were negative AmpC by M3D test. Twenty-eight out of 33 isolates were positive ESBL by MDDST. Twenty-six out of 33 were positive by double disc synergy test. The sensitivity of DDST was 92.9 % and specificity of 100% for detection of ESBL in absence of AmpC in the isolates.

This means that the sensitivity of the DDST when M3D was negative is better from its sensitivity when the latter was positive, where MDDST is positive in eight isolates more than double disc test in positive M3D isolates and two in negative M3D isolates.

Discussion

In our study, 62/100 isolates were positive by E-test. The DDST at a distance of 20mm center to center detected 52/62 (83.9 %) ESBL positive isolates by E-test with a sensitivity of 83.9% and specificity of 100 %. CAZ and CTX were found to be the best substrates, as they revealed synergism with AMC in 37/62 (59.7 %) and 35/62 (56.5 %) isolates respectively, followed by FEP which detected 21/62 (33.9 %). while the least detection was with CPD as it detected 11/62 (17.7 %). isolates. Also, Bamidele et al.[16] demonstrated that DDST using CTX, CAZ, CRO and ATM discs around AMC disc at a distance 15-20 mm center to center, on 54 isolates of *P. aeruginosa* collected from five different tertiary hospitals in Southwest Nigeria. Twenty-nine out of 54 were resistant to two or more expanded-spectrum cephalosporins. CRO and CTX were found to be the best substrate, as they revealed synergism with AMC in 29/54 (53.7 %) and 28/54 (51.8 %) respectively. On the other hand, ATM and FEP showed synergism in 24/54 (44.4 %) and 10/54 (18.6 %) isolates respectively, while CAZ showed the least synergy only in 9/54 (16.7 %).

In our study 67/100 isolates were AmpC producers by the M3D test. Thirty-four out of 67 AmpC producers were ESBL positive by E- test, and MDDST while by DDST 26/67 were ESBL positive showing 76.5 % sensitivity and 100 % specificity for detection of ESBL in the presence of AmpC. On the other hand, 26/33 (78.8 %) non-AmpC producers were positive by DDST Showing 92.9 % sensitivity and 100 % specificity of detection of ESBL in absence of AmpC. While, MDDST showed 100 % sensitivity and specificity in detection of ESBL in the presence and absence of AmpC as it identified the 10 isolates, which were not detected by DDST where MDDST was positive in eight AmpC producer isolates and the two AmpC negative isolates (i.e. all 62 ESBL positive isolates).

Similarly, Dhara et al.[15] demonstrated that 44/54 K*lebsiella* isolates collected from a blood culture from neonatal intensive care unit (NICU) patients referred to microbiology lab of B.J. Medical College, at Ahmedabad in India, were ESBL positive isolates by PCDDT and coproduce AmpC enzyme which was confirmed by the M3D test. MDDST detected the all 44 (100 %) ESBL positive isolates. Thus MDDST showed 100 % sensitivity and specificity for detection of ESBL in the presence of AmpC compared to the standard test DDST which detected 36/44 ESBL positive isolates.

In our study, 64/67 M3D-positive isolates were resistant to FOX with a sensitivity of (95.5 %) and a specificity of (63.6 %) in the detection of AmpC production. While 12/33 (36.4 %) M3D test negative was FOX resistant. While FOX susceptibility in three out of 67 (4.5 %) M3D-positive isolates may be explained by Peter-Getzlaff et al.[14] who stated that strains carrying ACC gene may appear FOX susceptible.

Conclusion

Double disc synergy test may be used for detection of ESBL in the isolates which produce only ESBL and not for detection of ESBL in isolates coproducing AmpC enzyme like *Enterobacter, Serratia, Citrobacter* and also with *Klebsiella spp.*

Modified double disc synergy test using TZP and FEP at a distance 20mm center to center may serve as a reliable confirmatory test for detection of ESBLs in AmpC positive isolates instead of DDST.

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