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Original Article

Microbiology Section

Modified Double Disc Synergy Test to Detect ESBL Production in Urinary Isolates of *Escherichia coli* and *Klebsiella pneumoniae*

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ABSTRACT

Background and Objectives: Various phenotypic methods are recommended in the routine practice to detect the ESBL production in gram negative bacilli. Among them, the Double Disc Synergy Test (DDST) which uses the third generation cephalosporins (3GC), is a simple and a reliable method. But the coexistence of AmpC may give false negative results. In such cases, the ESBL detection can be improved by using cefepime along with the third generation cephalosporins in DDST.

Methods: A total of 350 urinary isolates (224 *Escherichia coli* and 126 *Klebsiella pneumoniae*) were studied for ESBL production by the modified double disc test (MDDST) i.e. by using cefotaxime, ceftriaxone, cefpopdoxime (third generation cephalosporins) and cefepime (fourth generation cephalosporin) along with a amoxicillin-clavulanate disc.

Results and Interpretation: ESBL production was seen in 63.4% (142/224) *Escherichia coli* and in 60.3% (76/126) *Klebsiella pneumoniae* isolates by MDDST. Among these, in twelve *E.coli* and five *K.pneumoniae* strains, only cefepime but none of the third generation cephalosporins showed synergism with amoxicillin-clavulanate. All these seventeen strains showed a clear extension of the edge of inhibition which was produced by cefepime towards the amoxicillin-clavulanate disc. These strains were further tested for AmpC co-production by the AmpC disc test and all these strains were found to be AmpC positive, thus revealing the superior activity of cefepime in detecting ESBLs in the bacteria which co-produced AmpC. A high degree of co-resistance was found in the ESBL producers.

Conclusion: The ESBL detection can be improved by MDDST by using cefepime along with the third generation cephalosporins.

Key Words: Antimicrobial resistance, ESBL, Modified double disc synergy test

INTRODUCTION

The Extended Spectrum β-Lactamases (ESBLs) are typically plasmid-mediated enzymes that hydrolyze the penicillins, the third generation cephalosporins and aztreonam [1]. They are not active against the cephamycins (cephoxitin and cefotetan), but are susceptible to β-the lactamase inhibitors (clavulanic acid). The ESBL phenotypes have become more complex due to the production of multiple enzymes which include the inhibitor-resistant ESBL variants and plasmid-borne AmpC, the production of ESBLs in AmpC producing bacteria, the production of ESBLs in the KPC (*Klebsiella pneumoniae* Carbapenemase) producing bacteria, enzyme hyperproduction and porin loss [2,3]. These enzymes have spread worldwide and their prevalence varies by the geographical area. The extended spectrum β-lactamases often remain undetected by the current isolation and susceptibility methods. Molecular methods are the key tools for their detection but the facilities for them are not available in most of the laboratories, especially in the developing countries.

The clinical microbiological tests which are used for detecting ESBLs employ a β-lactamase inhibitor, usually clavulanate, in combination with the Third Generation Cephalosporins (3GC) such as ceftriaxone, ceftazidime or cefotaxime [4]. Recently, the coexistence of both AmpC and ESBLs in some gram negative organisms has been reported [3,5]. The AmpC β-lactamases are the cephalosporinases that are poorly inhibited by clavulanic acid and they can be differentiated from ESBLs by their ability to hydrolyze the cephamycins [6].

Such strains with the co-existing AmpC β-lactamases may give false negative tests for the detection of ESBLs, because clavulanic acid which is used in the standard DDST test for the ESBL detection acts as an inducer of the high level AmpC production and it leads to the resistance to the 3rd generation cephalosporins as well as to the 3rd generation cephalosporins + clavulanic acid. So, even if ESBL is present, it will not be detected and it may result in a false negative test. A modification in the original double disc synergy test i.e the Modified Double Disc Synergy Test (MDDST) with the use of the 4th generation cephalosporins (cefepime) and an optimum spacing of drugs for the detection of the synergy, depending on the zone of inhibition which is obtained with an extended-spectrum cephalosporin disc in a particular isolate, can improve the detection of ESBL in the strains which co-produce AmpC. A high–level AmpC production has a minimal effect on the activity of cefepime, thus making this drug a more reliable detection agent for ESBLs in the presence of an AmpC β-lactamase [7]. Tzelepi et al., reported a sensitivity of 16% only when the cefotaxime, ceftriaxone, ceftazidime and the aztreonam disks were used. The use of cefepime increased the sensitivity of the test to 61%, when the disk was placed at a standard distance (30 mm) from the clavulanate-containing disk. The sensitivity increased even

more, to 90%, when this distance was reduced to 20 mm [8]. The present study was therefore undertaken to evaluate the prevalence of the extended spectrum β-lactamase (ESBL) producing strains of *Escherichia coli* and *Klebsiella pneumoniae* in the urinary isolates at our tertiary care hospital by using the Modified Double Disc Synergy Test (MDDST).

MATERIALS AND METHODS

This study was conducted in the Department of Microbiology, Punjab Institute of Medical Sciences, Jalandhar, Punjab, India. A total of 350 isolates of *Escherichia coli* (224) and *Klebsiella pneumoniae* (126) which were obtained over a period of one year from January 2011 to December 2011, from patients with significant bacteruria, were identified, based on the colony morphology and the biochemical reactions. The clinico-demographic data of the study patients was noted. The Chi-square test was used to analyze the susceptibility pattern of the non β-lactam antibiotics in the ESBL producers and non-producers.

Antimicrobial Susceptibility Testing

The isolates were tested for their antimicrobial susceptibilities by the disc diffusion method according to the CLSI guidelines [8]. The following antibiotics were used; cefotaxime (30μg), cefpodoxime (30μg),ceftrioxone (30μg), ceftazidime (30μg), cefepime (30μg), cephoxitin (30μg), gentamicin (10μg), amikacin (30μg), ciprofloxacin (5μg), norfloxacin (10μg), nitrofurantoin (100μg), cotrimoxazole (25μg), piperacillin/tazobactam(100/10μg), meropenem (10μg), and imipenem (10μg). All the antibiotic discs were procured from Hi-media, Mumbai.

Testing for the ESBL Production

All the strains which showed a diameter of less than 27mm for cefotaxime and less than 25mm for ceftriaxone, were selected for checking the ESBL production. The ESBL production was tested by the Modified Double Disc Synergy Test (MDDST) by using a disc of amoxicillin-clavulanate (20/10 μg) along with four cephalosporins; 3GC-cefotaxime, ceftriaxone, cefpodoxime and 4GC-cefepime. A lawn culture of the organisms was made on a Mueller-Hinton agar plate, as was recommended by CLSI [9]. A disc which contained amoxicillin-clavulanate (20/10 μg) was placed in the centre of the plate. The discs of 3GC and 4GC were placed 15mm and 20mm apart respectively, centre to centre to that of the amoxicillin-clavulanate disc [10]. Any distortion or increase in the zone towards the disc of amoxicillin-clavulanate was considered as positive for the ESBL production. *Klebsiella pneumoniae* 700603 was used as a control strain for a positive ESBL production and *Escherichia coli* 25922 was used as a negative control for the ESBL production.

AmpC Detection [11]

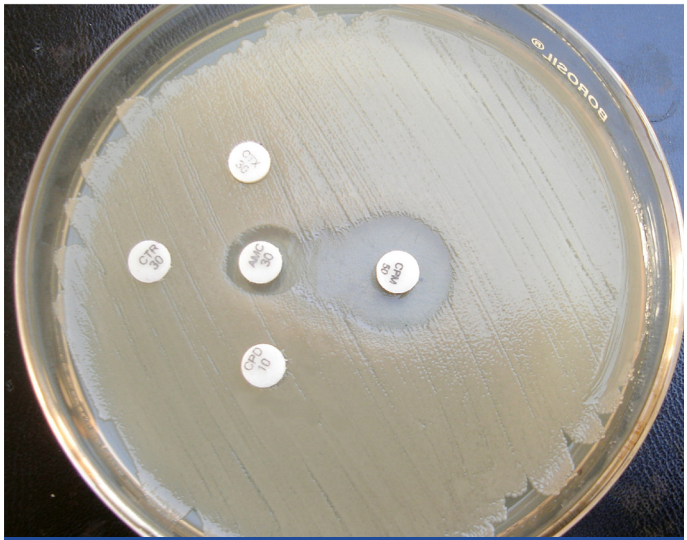
All the isolates which showed a synergistic effect with cefepime only in MDDST were further tested for the AmpC enzyme production by AmpC disc test after an initial screening with a cephoxitin (30 μg) disc. A lawn culture of a 0.5 McFarland's suspension of ATCC *E.coli* 25922 was prepared on a Mueller-Hinton agar plate. A 30 μg cephoxitin disc was placed on the inoculated surface of the agar. A sterile plain disc (6mm) which was inoculated with several colonies of the test organism was placed beside the cephoxitin disc, almost touching it. After an overnight incubation at 37°C, the plates were examined for either an indentation or a flattening of the zone of inhibition, which indicated the enzyme inactivation of cephoxitin (positive result), or an absence of distortion, which indicated no significant inactivation of cephoxitin (negative result).

RESULTS

Of the 350 isolates which were studied, 62.3% (218/350) were found to be ESBL producers by MDDST. ESBL production was seen in 63.4% (142/224) *Escherichia coli* and in 60.3% (76/126) *Klebsiella pneumoniae* isolates. Among these, in twelve *E.coli* and five *K.pneumoniae* strains, only cefepime but none of the third generation cephalosporins showed synergism with amoxicillin-clavulanate [Table/Fig-1]. All these seventeen strains showed a clear extension of the edge of inhibition which was produced by cefepime, towards the amoxicillin-clavulanate disk. [Table/Fig-2] The AmpC disk test was positive in all of these seventeen isolates [Table/Fig-3]. Eighty three percent (181/218) of the ESBL producers were from in-patients. These ESBL isolates were obtained from 90 male and 128 female patients, with a male female ratio of 1:1.4. They were distributed in the age group of 1year to 90 years. A false susceptibility to various β-lactam antibiotics was observed in the ESBL producers, the maximum susceptibility being to ceftazidime (13%) [Table/Fig-4]. A co-resistance to the non-β lactam antibiotics was observed more ($p<0.01$) with the ESBL producers [Table/Fig-5]. The resistance to amikacin and nitrofurantioin was comparatively less (17.4% and 13.3% respectively). Seventeen out of the 218 ESBL producing strains showed resistance to the combination drug, piperacillin/tazobactam. A resistance to the carbepenems is also emerging. The resistance to meropenem was observed to be more (6.0%) as compared to that to imepenem (3.2%).

Organism	Total ESBL producers*	ESBL+ AmpC Co-producers**	Pure ESBL producers
E.coli (n=224)	142 (63.4%)	12 (5.4%)	130 (58.0%)
K. pneumoniae (n=126)	76 (60.3%)	5 (4%)	71 (56.3%)
Total (n=350)	218 (62.3%)	17 (4.9%)	201 (57.4%)

[Table/Fig-1]: ESBL and AmpC production in *Escherichia coli* & *Klebsiella pneumoniae* isolates.
*Clavulanate synergy with either 3GCs or 4GC.
**Clavulanate synergy with only 4GC (cefepime) and none of 3GCs



[Table/Fig-2]: MDDST showing synergism of only cefepime but none of the 3 GC used with amoxicillin-clavulanate. (AMC–Amoxyclav, CTR-Ceftriaxone, CTX-Cefotaxime, CPD-Cefpodoxime, CPM- cefipime)

[Table/Fig-3]: Positive AmpC test (indentation of the zone of inhibition around cephoxitin disc)

Antibiotics	*E. coli* (n=142)	*K.pneumoniae* (n=76)	Total (n=218)
Ceftazidime	17	11	28(13%)
Cefotaxime	12	8	20 (9%)
Cefpodoxime	0	0	0 (0%)
Ceftriaxone	3	4	7 (3%)
Ccefepime	6	5	11(5%)

[Table/Fig-4]: In vitro susceptibility of ESBLs to β-lactam antibiotics

Antibiotics	ESBL producers (n=218) Susceptible No. %	Non-producers (n=132) Susceptible No. %
Norflox	15 6.9*	77 58.3
Ciprofloxacin	22 10.1*	85 64.4
Amikacin	180 82.6	122 92.4
Gentamicin	48 22.0*	114 86.4
Co-trimoxazole	39 17.9*	58 43.9
Nitrofurantoin	189 86.7	125 94.7
Piperacillin/Tazobactum	201 92.2	132 100
Meropenem	205 94.0	132 100
Imipenem	211 96.8	132 100

[Table/Fig-5]: Antibiotic susceptibility of ESBL producers and non Producers to non β-lactam various antibiotics
*p<0.01

DISCUSSION

The prevalence of ESBLs among the clinical isolates vary greatly world wide and in geographical areas and it is rapidly changing over time. In the west, the ESBL production in *Enterobacteriaceae varies* from 5 to 52 per cent and in other Asian countries, it varies from 10 to 46.5 per cent10 [9,12]. Other studies from India have reported a high prevalence of the ESBL production, which ranges from 41.0 to 63.6 per cent in *E. coli* and 40 to 83.3 per cent in *K. pneumoniae* [13-15]. ESBLs constitute a serious threat to the β-lactam therapy. Due to the difficulty in their detection by the current clinical methods, many of these strains have been falsely reported to be susceptible to the widely used broad-spectrum β-lactams [16]. The ESBL producers are intrinsically resistant to all the cephalosporins even if they exhibit an in vitro susceptibility [17]. In our study, false susceptibilities to ceftazidime and cefotaxime were observed in 13% and 9% of the ESBL producers. This could be due to the reason that the optimal substrate profile varies from one ESBL enzyme to another [18]. Hence, the susceptibility panels with only one extended spectrum cephalosporin cannot predict the resistance to the other extended spectrum cephalosporins [19]. The ESBL production coexists with the resistance to several other antibiotics. The ESBLs are encoded by plasmids which also carry resistant genes for other antibiotics [20]. A co-resistance to the quinolones and the aminoglycosides is common [21]. We found such an associated resistance with co-trimoxazole (82.1%), gentamicin (78%) and the flouroquinolones (90-93%). Varsha et al reported 91.17%, 100% and 94.91% resistances respectively to gentamicin, cotrimoxazole and ciprofloxacin in the ESBL producers [22]. The high resistance to the non β-lactam antibiotics of the ESBL producing strains poses a threat of treatment failure by these drugs and it also minimizes the therapeutic choice to the carbapenems. Hence, the emerging resistance to the carbepenems is a phenomenon of great concern for combating the infections of the multidrug resistant bacteria [23]. Although β-lactam/ β- lactamase inhibitor combinations have been suggested as the treatment option for ESBL producers, these drugs must be given in high doses [24].

The ESBL testing in the AmpC-producing species of *Entero-bacteriacae* is an unresolved issue. In the presence of AmpC, along with ESBL in the gram negative organisms, the DDST may not show positivity, as the AmpC type of β-lactamase inhibits the action of clavulanate. Hence, it obscures the synergistic effect of clavulanic acid and the 3GCs which are used. The possible approaches for overcoming the difficulty in the ESBL detection in the presence of AmpC include the use of tazobactam or sulbactam, which are much less likely to induce the AmpC β-lactamases and are therefore the preferable inhibitors of the ESBL detection tests with these organisms or testing cefepime as an ESBL detection agent [6]. Cefepime, a fourth –generation cephalosporin, is a more reliable detection agent for ESBLs in the presence of the AmpC β-lactamases, as this drug is stable to AmpC β-lactamase. Thus, it will demonstrate the synergy which arises from the inhibition of ESBL by clavulanate in the presence of the AmpC enzyme. Previously, Tzouvelekis et al., and Tzelepi et al., had reported that the use of cefepime had increased the sensitivity of DDST with the extended spectrum cephalosporins for the detection of ESBLs in *Klebsiella pneumoniae* and the *Enterobacter species* respectively [25,8]. In another study, the performance of a Modified Double-Disk Test (MDDT) which utilized cefotaxime, ceftazidime, cefepime and aztreonam along with a amoxicillin-clavulanate disk, was evaluated for the detection of ESBLs in the clinical isolates of *E.coli* and *K.pneumoniae* [26]. Of the 136 isolates, 112(82%) and 102(75%) were positive for ESBL by the MDDT and the NCCLS/CLSI methods respectively. Ten (7.4%) isolates (eight *E.coli* and two *K.pneumoniae*), all of which were positive for ESBL by MDDT, yielded negative results with the NCCLS/CLSI disk method [26]. In another study, two *K. pneumoniae* isolates out of 100 consecutive

isolates of *E. coli* and *Klebsiella,* were positive by the double-disk synergy test for ESBL with cefepime only, but not with any of the other third-generation cephalosporins which were used [22]. A study of MKR Khan et al., had similar findings, in which DDST was positive in 25/40 isolates and MDDST was positive in 40/40 isolates [27]. Mohanty et al evaluated the performance of the cefepime/ cefepime-clavulanate ESBL E test for detecting the ESBL production in *E.coli*, *K.pneumoniae* and *P.mirabilis,* which detected the maximum number of isolates (70/113, 61.9%) as ESBL positive as compared to the ceftazidime/ceftazidime-clavulanate and the cefotaxime/cefotaxime-clavulanate strips, which detected 57 (50.4%) isolates each as ESBL positive. In the 66 (58.4%) isolates that co-produced AmpC, in addition to the ESBL enzymes, the cefepime/cefepime-clavulanate E test strip detected ESBLs in an additional 13(11.4%) isolates as compared to the other ESBL E test strips [3]. More recently. Dhara et al., demonstrated the superiority of MDDST over DDST for the ESBL detection [28]. So, 4GC-cefepime has been recommended as an alternative cephalosporin for the ESBL detection in the presence of AmpC. In our study, we used both 3GC and 4 GC in MDDST. 4 GC-Cefepime helped in detecting seventeen additional strains as ESBL producers, which otherwise would have gone undetected with the use 3GC alone, as in the original double disc synergy test.

CONCLUSION

The frequency of the ESBL production can easily be underestimated in the clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* with the use of the current CLSI [10] recommended methods, since these organisms often produce multiple β-lactamases. In such situations, where the AmpC β-lactamases can interfere with the clavulanate synergy, the application of the double disc synergy tests that combine amoxicillin-clavulanate with cefepime, may increase the possibility of the ESBL detection .An optimum identification of the ESBL producing isolates is essential to formulate policies for an empirical antimicrobial therapy, especially in high-risk units where the infections which are caused by these organisms are common. It also helps in monitoring the development of antimicrobial resistance and in the implementation of proper hospital infection control measures.

Contribution:

1. Conducted the Reseach Work,
   Data Analysis and Iterpretation, Drafted the Article.
2. Helped the Research and Drafting of the Article.
3. Data Analysis and Interpretation, Edited the Article.
4. Data Analysis and Interpretation, Edited the Article.

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