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Detection of Extended Spectrum Beta Lactamases from Clinical Samples

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ABSTRACT

Background and objective Extended spectrum beta-lactamases (ESBLs) are a rapidly evolving group of beta-lactamase which share the ability to hydrolyze third generation cephalosporins and aztreonam but are inhibited by clavulanic acid. Their detection is essential for proper antibiotic therapy, to limit the spread of resistance mechanisms and for epidemiological purposes. This study was to determine the detection of ESBL to know the best suitable one in our lab setup.

Material and method: The total of 53 Gram negative isolates were identified based on the different biochemical test as per the standard protocol and Antibiotic susceptibility testing (AST) was done. ESBL production was observed which were resistant to one of the third generation cephalosporins were selected provisionally as ESBL producers and then subjected for confirmation by Phenotypic confirmatory Double Disk Synergy Test (30 mm), Double Disk Synergy Test (20 mm), Modify Double Disk (20), ESBL E-Test to evaluate their ability to detect ESBLs.

Result: In case of ESBL E test the 16 ESBL production was observed and highest sensitivity was observed in case of Cefepime only. In case of DDST (30mm) 14 ESBL production was observed and specificity in cefepime was 82% and further increased upto 84% in case of cefepime combination. DDST (20mm) 15 ESBL production was observed again the sensitivity was high for Cefepime alone (87%), while in combination it was 87% and 89% with Ceftazidime and Cefotaxime. MDDST (20mm) identified 16 isolates as ESBL, resulting the sensitivity of 91%.

Conclusion: In this study we noticed that E. coli shows maximum ESBLs production and cefepime is most effective cephalosporins for detection of ESBL followed by cefotaxime, ceftazidime and cefpodoxime. This sensitivity of the test can be further improved with MDDST.

Key Words: ESBL, DDST, MDDST, E-Test

1. Introduction

Few organisms of Enterobacteriaceae family are able to produce an enzyme called Extended Spectrum beta-Lactamase (ESBLs) that are rapidly evolving, plasmid encoded enzymes that have ability to hydrolyze all Beta lactam antibiotics including cephalosporins and azoteronam but are inhibited by clavulanic acid. Penicillinase, the oldest beta-lactamases, although being sensitive to third generation cephalosporins, conferred resistance to antibiotics like cephamycins, penicillin and carbapenems.

However, ESBLs mediate resistance to extended spectrum cephalosporins (third generation cephalosporins, 3GCs) and monobactams with the exception of cephamycins or carbapenems **(Paterson et al, 2005).** ESBLs are derived from mutation in the older beta-lactamase TEM, CTX-M and SHV gene by one or more amino acid substitutions around the active site, in which CTX-M is the most common ESBL type worldwide **(Paterson et al., 2005).** Beta-lactams acts primarily as inhibitors of the synthesis of the cell wall, by blocking the action of transpeptidases by the covalent attachment to penicillin-binding protein (PBP), which is a peptidoglycan transpeptidase enzyme responsible for catalyzing the final steps in cell wall. After blocking the active site of these enzymes, betalactam antibiotics deprive them of their natural substrates, thus preventing the cross linking of cell wall and ultimately resulting in cell death due to osmotic instability **(Pitton JS, 1972)** Beta-lactam antibiotics derive their name from having a beta-lactam ring in their structure, through the addition of one activated water molecule, beta-lactamases enzyme carries out destruction of that beta-lactam ring. The point mutation in beta-lactamases introduced serine in the active site of ESBLs, causing their attack on the amide bond in the lactam ring of antibiotics, resulting in their hydrolysis **(Chaudhary and Aggarwal, 2004**).

2. Materials and Methods

2.1 Sample collection and handling

Total 150 clinical samples were collected. The clinical samples including urine, pus, sputum, bal, ET, TT, swab or intravenous cather were obtained from patient units of medicine, surgery, gynecology and obstetrics, pediatrics and intensive care unit (ICU) over a period of 90 days. The samples were collected irrespective of age and sex. The samples were processed and isolates were characterized following standard laboratory procedures. **(Bergey's, 1985)**

2.2 Isolation and characterization

For the isolation of organisms all 150 clinical samples were inoculated first on Mac-conkey's agar media and incubate at 37 for 24 h. After incubation culture characteristics of isolates were identified based on various biochemical characterizations as per standard microbiological techniques **(Bergey's, 1985).**

2.3 Antibiotic susceptibility testing

The isolates were tested for the antimicrobial susceptibilities by the disc diffusion technique on Muller-Hinton agar according to the second edition of Bergey's Manual of Systematic Bacteriology **(Prescott et al 2003).** The turbidity of inoculums suspension was adjusted to 0.5 Macfarland's standard. Then this suspension was inoculated onto Muller-Hinton agar plate by lawn culture. After that, the antibiotic discs were placed using sterile forceps and pressed gently to confirm proper contact with medium. The plates were then incubated at 37 for 24 hrs. The zone of inhibition was measured and interpreted as per CLSI guideline **(CLSI 2008).**

2.4 Detection of ESBLs

2.4.1ESBLsscreeningtest

Screening methods involve detection of resistance to any of the third-generation cephalosporin antibiotics such as cefotaxime, ceftazidime, ceftriaxone, cefpodoxime or aztreonam by disk diffusion

method or by MIC estimation. The test inoculum (0.5 McFarland's turbidity) was spread onto the MHA by using a sterile cotton swab. According to the CLSIs guidelines, isolates showing inhibition zone size of ≤ 22 mm with Ceftazidime (30 µg), ≤ 25 mm with Ceftriaxone (30 µg), ≤ 27 mm with Cefotaxime (30 µg) , ≤ 27 mm with Aztreonam (30 µg) and ≤ 22 mm with Cefpodoxime (10 µg), were identified as potential ESBL producers and short listed for confirmation of ESBL production. The isolates which showed resistance to any one of these antibiotic discs were considered as screen positive. These isolates were further tested for ESBL production by Double disc synergy test-30mm (DDST) and Double disc diffusion test -20mm (DDST) method.

2.5 Confirmatory tests for ESBLs

2.5.1 Double Disk Synergy Test (30mm)-

The isolated organisms which were screened positive for ESBL production were tested by DDST. The turbidity of inoculums suspension was adjusted to 0.5 Macfarland's standard was spread onto the MHA. The double-disk synergy method at 30 mm (DDS30) is integrated as an adjunct of the routine susceptibility test by the disk diffusion method (Bradford PA). CTX (30 µg), CAZ (30 µg), CTR (30 µg) and CPD (10 µg) disks (Hi-Media) were placed on the Mueller-Hinton (MH) agar plates at a distance of 30 mm, center to center, from either a Piperacillin/tazobactum (P/T; 20 and 10 µg) disk and incubated at 35 ^{0}C for 18 to 48 hours. The presence of ESBL was inferred when the inhibition zone around any of the four antibiotic disks was enhanced on the side of the tazo-containing disk, resulting in a characteristically shaped zone referred to as a "champagne-cork," "keyhole," "ellipsis," or "phantom image."

2.5.2 Double Disk Synergy Test(20mm)-

An amoxicillin-clavulanate disk was manually placed at 20 mm, centre to centre, of CTX, CAZ, CTR, and CPD disks on MH agar plates. Interpretation criteria for ESBL production were similar as those described above double disk synergy test (30mm) **(Winokur *et al* 2001, Paterson *et al* 2005, Moghaddam *et al* 2012).** Enhancement of zone of inhibition towards Piperacillin/tazobactum by any of these antibiotic discs was considered as ESBL producer.

2.5.3 Modified Double Disc Synergy Test-

A total of micro-organisms isolates were studied for ESBL production by the modified double disc test (MDDST) i.e. by using cefotaxime, ceftriaxone, cefpodoxime (third generation cephalosporins) and cefepime (fourth generation cephalosporin) along with a amoxicillin-clavulanate disc. The plates were incubated at 37 for 24 h. fourth generation cephalosporin is unaffected by AmpC beta-lactamases.

2.5.4 ESBL E-Test-

The E test ESBL strip (AB Biodisk, Solna, Sweden) carries two gradients: on the one end, ceftazidime; and on the opposite end, ceftazidime plus clavulanic acid. **(Pangon *et al* 1989)** MIC is interpreted as the point of intersection of the inhibition ellipse with the E test strip edge. A ratio of ceftazidime MIC to ceftazidime-clavulanic acid MIC equal to or greater than 8 indicates the presence of ESBL. The reported sensitivity of the method as a phenotypic confirmatory test for ESBLs is 87% to 100%. **(Pangon *et al* 1989, Bush *et al* 1989)** and the specificity is 95% to 100%. The availability of

cefotaxime strips, as well as ceftazidime strips, improves the ability to detect ESBL types, which preferentially hydrolyze cefotaxime, such as CTX-M–type enzymes. (Bradford PA)

RESULTS:

During this study period, 150 different clinical samples were collected from tertiary care hospital, of which 53 (42%) samples showed significant growth. Gram staining and series of biochemical tests were performed for the characterization of the isolates. They were identified as *Escherichia Coli* (n=29), *Klebsiella pneumoniae* (n=16), *Pseudomonas aeruginosa*(n=6), and *Atypical E.Coli* (n=2). ESBL production was observed in 15 isolates.

In case of Double Disk Synergy Test 30 mm, among all the four beta lactams highest sensitivity and specificity was observed in Cefepime as 82% and 100% respectively. The sensitivity was further increased up to 84% in case of Cefepime combination, however the specificity remained same. Maximum number of false negatives was also observed in this method (Table-2).

In case of Double Disk Synergy Test 20 mm, again the sensitivity was high for Cefepime alone (87%), while in combination it was 87% and 89% with Ceftazidime and Cefotaxime.

False negative is less in comparison to Double Disk Synergy Test 30 mm.

Modified double disk synergy test (20mm) identified 15 isolates as ESBL, this method able to pick up one more isolates of E. coli which was not picked by other method, resulting the sensitivity of 92%. (Table-3). In case of ESBL E test the highest sensitivity was observed in case of Cefepime only.

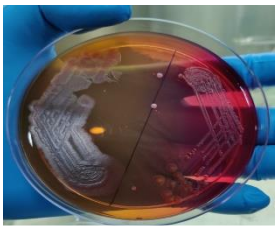

Fig.1 Growth on Mac-Conkey Agar

Fig.2 Biochemical Test for Identification

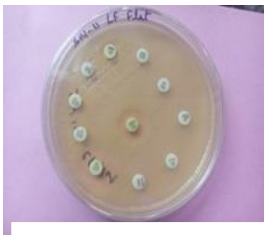

Fig.3 ESBL Screening Test

Fig.4 ESBL Screening Test

Fig.5 Double Disk Synergy Test

Fig.6 ESBL E Strip Test

Table1 : Distribution of ESBL isolates among the phenotypes methods

Method	DDST (30 mm)		DDST (20 mm)		MDDST		ESBL Etest	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative

No. of ESBL Positive Isolates	12	3	13	2	15	1	14	1
No. of ESBL Negative Isolates	2	36	2	36	1	36	2	36
Total	14	39	15	38	16	37	16	37

Figure 7: Graph showing distribution of ESBL among tested organism

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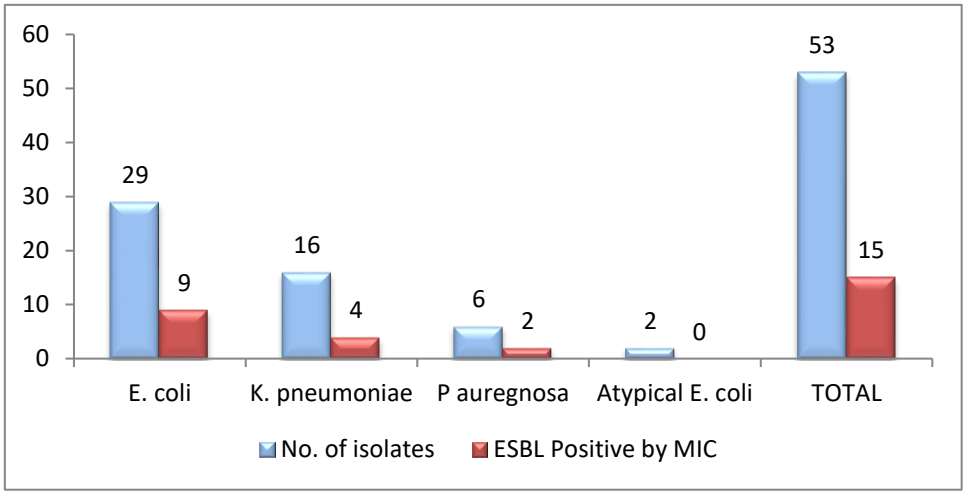

Table 2: Statistical analysis of the various parameters of Phenotypic methods

Antibiotics	**All Isolates (53)**			
	Sensitivity (%)	**Specificity (%)**	**PPV (%)**	**NPV (%)**
Double Disk Synergy Test (30 mm)				
Ceftazidime	79	100	100	92
Cefotaxime	80	100	100	92
Cefpodoxime	74	100	100	90
Cefepime	82	100	100	93
Ceftazidime + Cefotaxime	82	100	100	93
Ceftazidime + Cefepime	84	100	100	92
Cefotaxime + Cefepime	84	100	100	94
Double Disk Synergy Test (20 mm)				
Ceftazidime	82	100	100	93
Cefotaxime	84	100	100	94
Cefpodoxime	77	100	100	91
Cefepime	87	100	100	95

Ceftazidime + Cefotaxime	85	100	100	94
Ceftazidime + Cefepime	87	100	100	95
Cefotaxime + Cefepime	89	100	100	96
Modified Double Disk Synergy Test (20 mm)				
Cefpodoxime, Ceftazidime, Cefotaxime &Cefepime with piperacillin/tazobactam at centre	91	100	100	97
ESBL Etest				
Ceftazidime	84	100	100	93
Cefotaxime	86	100	100	95
Cefepime	92	99	98	96

DISCUSSION-

This study demonstrates the presence of ESBL-mediated resistance in gram-negative bacilli causing infections in various wards and ICU of a tertiary hospital in Raipur. Although a few studies have reported on the prevalence of ESBL producers in Indian hospitals, ESBL producing bacteria may have evolved in several hospitals all over the country. ESBL detection is not commonly carried out in many microbiology units in developing countries, India included. This could be attributed to lack of awareness and lack of resources and facilities to conduct ESBL identification. The high rate of resistance noted among the isolates in the present study, is of serious concern indicating multidrug resistance pattern. Mechanisms of co-resistance are not clear, but one possible mechanism is the co-transmission of ESBL and resistance to other antimicrobials within the same conjugative plasmids.

It has been previously reported that combination disk of cefotaxim and ceftazidime has a high sensitivity for detection of ESBL in clinical samples **(Wiegand *et al*.,2007).** Thompson and Sanders, 1992 used the recommended disk spacing of 30mm and then repeated at 20mm to see if the former disk spacing was negative. In this study in case of double disk synergy test 30 mm, it was observed that combination of cefotaxim and ceftazidime has achieved the sensitivity of 82%, but when Cefepime was used in combination the sensitivity was further improved upto 84%. However, in case of double disk synergy test 20 mm the sensitivity of cefotaxim and ceftazidime combination was comparative more than 20 mm test, it was 86% which was further increased upto 89% when cefepime was used in combination. This observation of increased sensitivity for ESBL detection with Cefepime combination was also reported by **A.J. Linscott and Brown, 2005**.

Jarlier *et al*., 1988; Thompson and Sanders, 1992 had reported previously that modified double disk synergy test increase the sensitivity of ESBL detection. Observed the sensitivity of 92% in this method when piperacillin-tazobactam was kept in centre and other disk was kept 20mm apart. Used piperacillin-tazobactam inspite of ampicillin-clavulinic acid because it has already reported that clavulinic acid may induce the high-level expression of AmpC production in the organism producing ESBL and AmpC together, and may antagonize rather than protect the antibacterial activity of patner betalactam, thereby masking the synergy arising from inhibition of ESBL.

In case of E-test, as per manufacture recommendation, Cefotaxime and Ceftazidime is the first line method for ESBL detection. In our case comparative high sensitivity was observed with Cefepime.

In this study, we noticed that Cefepime is most effective cephalosporin followed by cefoitaxim, ceftazidime and cefpodoxime This sensitivity can be further improved with modified double disk synergy test (20mm), similar findings were observed by **Sturenberg *et al*., 2004; Garrec *et al*., 2011**. However, **Cormician *et al*.,1996** shows maximum detection of ESBL by Ceftazidime.

SUMMARY:

In this study, noticed that E. coli shows maximum ESBLs and Cefepime was found to be most effective cephalosporin followed by cefotaxim, ceftazidime and cefpodoxime This sensitivity can be further improved with modified double disk synergy test (MDDST).

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