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DETECTION OF EXTENDED-SPECTRUM BETA-LACTAMASE PRODUCTION IN GRAM NEGATIVE BACTERIA FROM DIFFERENT CLINICAL ISOLATES

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ABSTRACT

361 gram negative isolates were identified from different clinical specimens (urine, sputum, pus, blood, ET tip, and pleural fluid) collected from patients at two tertiary care hospitals in Hyderabad. The predominant bacterial isolates were *Escherichia coli* and *Klebsiella* species. The screening test detected 174(48.19%) of the 361 isolates as potential Extended-spectrum beta-lactamase (ESBL) producers. Out of the 174 isolates which tested positive in the screening test, 137 (38%) were positive in both confirmatory tests, the phenotypic confirmatory combination disc diffusion test and the Epsilometer test, proving that both tests were equally effective. Multidrug resistance was seen in 127 (92.7%) of ESBL positive isolates and 94 (41.9%) of the non ESBL isolates, and the difference was statistically significant ($p < 0.01$). Because of the high resistance pattern of ESBL producers, it is imperative to detect them in order to overcome therapeutic failures.

KEYWORDS: **Extended-spectrum beta-Lactamases (ESBL), Gram-Negative Bacteria, Multidrug resistance**

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INTRODUCTION

Resistance to various antibiotics is a problem faced in clinical therapeutics from the time Penicillin was introduced as an antibiotic. Among the wide array of antibiotics, beta-lactam drugs (Penicillins, Cephalosporins, Monobactams and Carbapenems) are the most widely used agents accounting for greater than 50% of global antibiotic consumption[1,2]. Production of beta-lactamase is the most common cause of resistance to beta-lactam antibiotics. The persistent exposure of bacterial strains to the multitude of beta-lactams has induced the production of new beta-lactamases, one emerging for each new class of antibiotics, with expanding activity even against the third and fourth generation Cephalosporins such as Ceftazidime, Cefotaxime, Cefpodoxime, Cefepime and Aztreonam. These new beta-lactamases are called Extended-spectrum beta-lactamases. The Extended-spectrum beta-lactamase (ESBL) enzymes are encoded by genes that are typically plasmid borne. ESBL's hydrolyse Penicillins, Cephalosporins and Aztreonam and are inhibited by Clavulanic acid.[3,4] The ESBL producing bacteria are typically associated with multidrug resistance because genes with other mechanisms of resistance often reside on the same plasmid as the ESBL gene. Thus some ESBL producing strains also show resistance to Quinolones, Aminoglycosides and Antifolates.[5,6,7] .As they are plasmid mediated, the resistance can spread fast among gram negative bacteria. Because of the high prevalence of resistance among ESBL producers, detection of ESBL production is of major clinical concern. Inappropriate treatment of invasive infections with Cephalosporins can lead to therapeutic failures and adverse clinical outcomes, and worse still, nosocomial out breaks of infections because of ESBL producing gram negative bacteria. ESBL's are found in significant percentage of *Escherichia coli* and *Klebsiella* strains. They have also been found in *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterobacter, Serratia marcescens*, *Citrobacter, Morganella morganii, Shigella dysenteriae* and *Burkholderia cepacia*[3,8,9,10,11,12] .Studies from India have reported ESBL production varying from 6% to 68%.[13] The increased prevalence of bacterial pathogens producing ESBL'S requires laboratory testing methods for their accurate and rapid detection in clinical isolates.[14]Several phenotypic and genotypic methods are present. Genotypic methods are based on enzyme detection by PCR which is not suitable for routine clinical testing. Phenotypic methods guided by Clinical Laboratory Standards Institute (CLSI) are most widely accepted and used, with good clinical outcomes and were adopted in the present study.

An ESBL is specifically inhibited by a beta-lactamase inhibitor such as Clavulanic acid and this property is utilized for the detection and confirmation of ESBL's.[15]In the present study screening test was done to identify potential ESBL producers using Ampicillin, Cefodroxil, Cefotaxime, Ceftazidime, Aztreonam and Imipenem discs, and those identified were short-listed for phenotypic confirmatory combination disc diffusion test and E-Test. Finally antibiotic susceptibility patterns of ESBL-producers and non-ESBL producers were compared to understand the magnitude of the problem and to implement appropriate therapeutic measures. Accurate detection of ESBL is critically important for manifold reasons. First, ESBL production is a worldwide problem of increasing prevalence. It is also crucial to identify strains that produce ESBL, as these are also multidrug resistant. Finally, it is important to find out the occurrence of these multiple resistant strains in individual clinical setup so as to avoid antibiotic treatment failures in critical clinical emergencies.

MATERIALS AND METHODS

The present study was conducted in the department of Microbiology, from June 2008 to May 2009. Samples were collected from the in-patient and out-patient units of Princess Esra Hospital and Owaisi Hospital and Research Centre, Deccan College of Medical Sciences.

The specimens collected were urine, sputum, pus swab, blood and others which included pleural fluid, liver aspirate, tracheostomy tube tip, endotracheal suction catheter tip, central line catheter tip etc., from both genders and all age groups. Samples were processed on MacConkey's agar, Blood agar and Nutrient agar and incubated at 37^0C for 24hours. After 24 hours, plates were observed for growth and all isolates were identified by standard microbiological procedures. Antimicrobial sensitivity test was carried out by Kirby-Bauer disc diffusion method using Mueller Hinton agar and results interpreted according to CLSI guidelines.

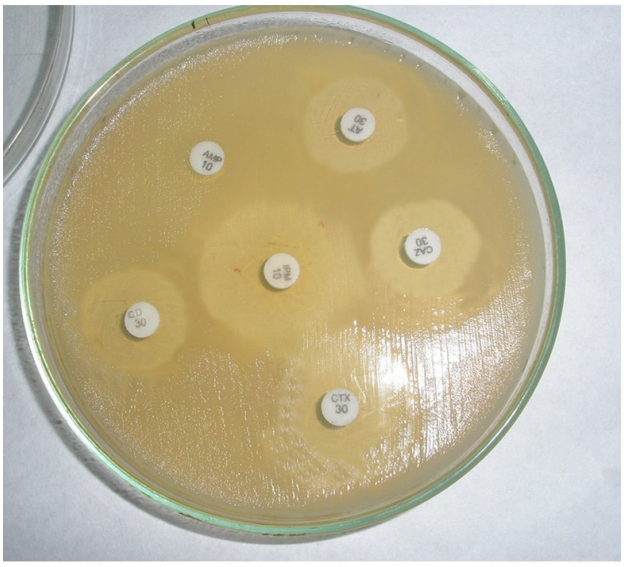
ANTIBIOTIC DISCS

Readymade antibiotic discs procured from Himedia Mumbai were used. They were Ampicillin (10µg), Amikacin(30µg), Gentamicin (10µg), Ciprofloxacin (5µg), Cefepime (30µg), Cefadroxil(30µg), Ceftadizime(30µg), Cefotaxime(30µg), Imipenem (10µg), Piperacillin-tazobactam (100µg/10µg),Cefoxitin(30µg), Aztreonam(30µg), Cefoperazone-Sulbactam(75/10µg), Ceftadizime-Clavulanic acid (30/10µg), Cefpodoxime(10µg), Meropenem(10µg), Cefuroxime(30µg), Ceftriaxone (30µg), Cefoperazone (75µg), Amoxyclav (20/10µg).

SCREENING TEST FOR IDENTIFICATION OF POTENTIAL ESBL PRODUCERS

As per CLSI guidelines, the screening test was carried out by Kirby-Bauer disc diffusion method on Mueller Hinton Agar, using antibiotic discs such as Ampicillin (10µg), Cefadroxil (30µg), Cefotaxime (30µg), Ceftazidime (30µg), Aztreonam (30µg) and Imipenem (10µg)[16]. The use of more than one of the agents for screening improves the sensitivity of the detection. The results were interpreted using the following zones of inhibition: Ceftazidime<27mm, Aztreonam<15mm, Cefadroxil<14mm, Ampicillin<13mm, and Imipenem<16mm. If the isolate was found resistant either to Cefotaxime, Ceftazidime or Aztreonam, it was considered as potential ESBL producer and further subjected to phenotypic confirmatory tests to ascertain the diagnosis.

FIGURE -1 : Screening Test for an Extended Spectrum β - Lactamase



Screening test of an ESBL - producing strain showing resistance to Ampicillin (AMP), Cefatoxime (CTX), Cefadroxil (CD) Aztreonam (AT), Ceftazidime (CAZ); and sensitivity to Imipenem (IPM).

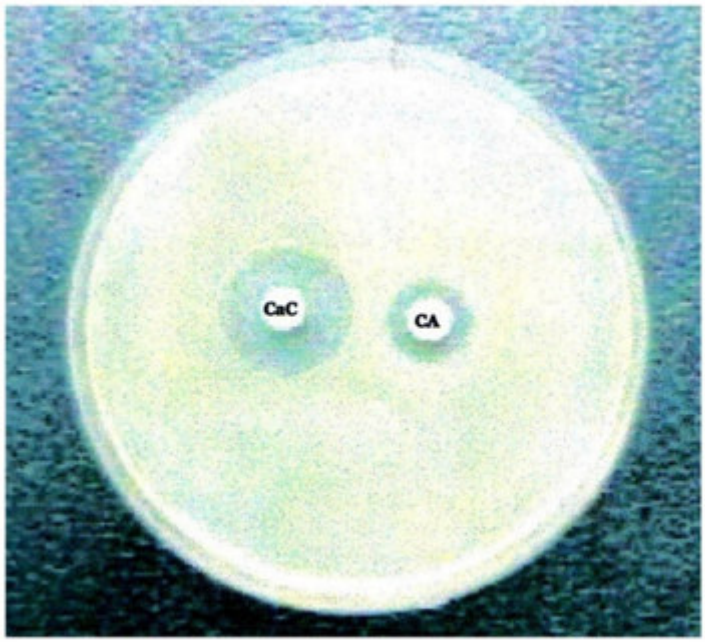
PHENOTYPIC CONFIRMATION TESTS

All the test isolates which were positive in the screening test were subjected to the Phenotypic confirmatory combination disc diffusion test (PCCDDT) and Epsilometer Test (E-test).

1) Phenotypic confirmatory combination disc diffusion test (PCCDDT)

Ceftazidime (30 µg) and Ceftazidime - Clavulanic acid (30/10 µg) discs were placed at a distance of 25mm apart from centre to centre on the lawn culture of the test isolate on Mueller Hinton Agar plate, and incubated at 35^0C for 18 to 24 hours. After incubation the zone differences in zone diameter, with or without Clavulanic acid, were measured. A positive result was indicated when there was an increase in >=5mm inhibition zone diameter around combination disc of Ceftazidime + Clavulanic acid versus the inhibition zone diameter around Ceftazidime disc alone, which confirmed ESBL production.

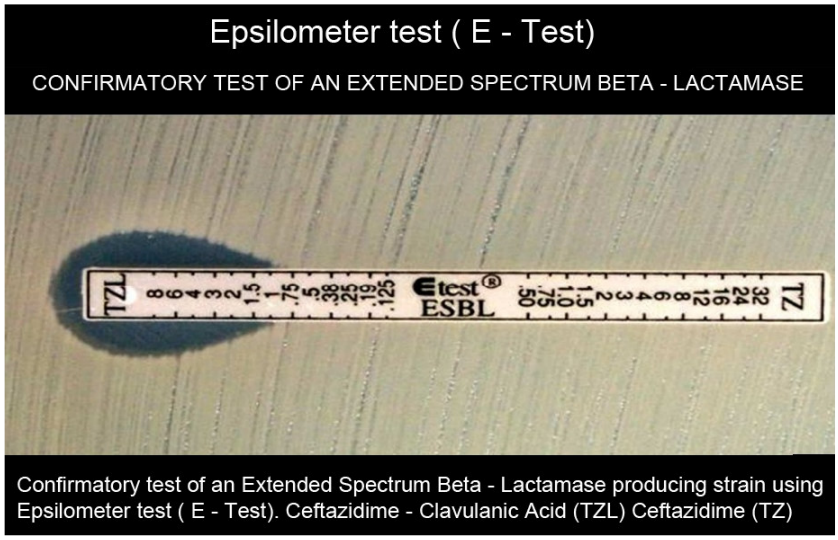
FIGURE 2: Confirmatory Test of an Extended-Spectrum β - Lactamase



Phenotypic confirmatory test of an Extendend Spectrum β - lactamase - producing strain using combination disk; Ceftazidime - Clavulanic acid (CaC) (30 µg/10 µg), and Ceftazidime (CA) (30 mg) alone

2) Epsilometer Test (E-Test)

Confirmation of ESBL production was also done by E-test procured from Bio-Merieux, France. The test was performed in accordance with the guidelines of the manufacturer. In this method, a 90mm Mueller Hinton Agar plate was taken, 0.5 Mc Farlands standardized inoculum of the test organism was swabbed and allowed to dry at room temperature. An E-test strip with Ceftazidime at one end, and Ceftazidime + Clavulanic acid at the other end in a concentration gradient, was placed on the lawn culture plate and incubated at 35^0C for overnight. After overnight incubation, results were interpreted. The minimum inhibitory concentration (MIC) was interpreted as the point of intersection of the inhibition ellipse of bacterial growth with the MIC gradient on the E-test strip. The lowest concentration gradient which inhibits the bacterial growth is the MIC of the drug. The ratio of the Ceftazidime MIC and Ceftazidime + Clavulanic acid MIC equal to or greater than 8 indicated the presence of ESBL. The presence of Extended-spectrum beta-lactamases was confirmed by the appearance of phantom zone below, or deformation of Ceftazidime inhibition ellipse or when Clavulanate caused a more than or equal to three doubling concentration decrease (ratio of more than 8) in the MIC values of Ceftazidime.



Confirmatory test of an Extended Spectrum Beta - Lactamase producing strain using Epsilometer test (E - Test). Ceftazidime - Clavulanic Acid (TZL) Ceftazidime (TZ)

RESULTS

A total of 361 gram negative bacterial isolates recovered from different clinical samples from patients of both in-patient and out-patient units during a one year period (June 2008 to May 2009)were processed in the department of Microbiology, Deccan College of Medical Sciences and studied. Total number of samples which yielded gram negative bacterial isolates were 361.Of these, 179 (49.58%) were from urinary samples, 83 (22.99%) were from pus samples, 62 (17.17%)were from sputum samples and 16 (4.43%) were blood culture isolates. Others, which included catheter tips, endotracheal suction catheter tip, tracheostomy tube tip and exudates like pleural effusion and liver abscess fluid, constituted 21 (5.81%). *Escherichia coli* was the most frequently isolated, comprising 153 (42.38%), followed by *Klebsiella* species at 126 (34.90%), *Pseudomonas aeruginosa* 65 (18%), *Acinetobacter* species 8 (2.21%) and *Proteus mirabilis* 6 (1.66%). In addition, 1 each of *Enterobacter*species, *Citrobacter* species and *Salmonella typhi* constituting 0.27% were the remaining isolates. (Table – 1)

Table 1
Frequency of occurrence of gram negative bacterial isolates

Organism	Total number of isolates	% of total isolates
Escherichia coli	153	42.38
Klebsiella species	126	34.90
Pseudomonas aeruginosa	65	18
Acinetobacter species	8	2.21
Proteus mirabilis	6	1.66
Entterobacter species	1	0.27
Citrobacter species	1	0.27
Salmonella typhi	1	0.27
Total	361	

The screening test for ESBL production was conducted for all the 361 isolates according to CLSI guidelines.[16]The screening test detected 174 (48.19%) as potential ESBL producers. Out of 174, *Escherichia coli* with a count of 174 (48.85%) were maximum, followed by *Klebsiella* species 58 (33.33%), *Pseudomonas aeruginosa* 21(12.08%), *Acinetobacter* species, 6 (3.44%) and *Proteus mirabilis*, 4 (2.3%). *Enterobacter* species, *Citrobacter* species and *Salmonella typhi* were negative in the screening test and therefore non-ESBL producers. Comparing the two confirmatory tests for ESBL production, both showed equal numbers of positives. The

study revealed the same efficacy of both methods in detecting ESBL production. (Table – 2)

Table 2
Results of screening and confirmatory tests for ESBL

Organism	Total No. of isolates	No. of Isolates positive in Screening test	No. of positive Isolates in PCCDDT	No. of positive Isolates in E-test
Escherichia coli	153	85(55.55%)	70(45.75%)	70(45.75%)
Klebsiella species	126	58(46.03%)	48(38.09%)	48(38.09%)
Pseudomonas aeruginosa	65	21(32.30%)	15(23.07%)	15(23.07%)
Acinetobacter species	8	6(75%)	2(25%)	2(25%)
Proteus mirabilis	6	4(66.67%)	2(33.33%)	2(33.33%)
Total	361	174(48.19%)	137(38%)	137(38%)

Total number of isolates producing ESBL were 137 (38%) out of 361, as revealed by the confirmatory test results. *Escherichia coli* isolates were maximum, showing, 70/137 (51.09%), followed by *Klebsiella* species 48 (35.04%), *Pseudomonas aeruginosa* 15 (10.95%) and 2 (1.46%) each of *Acinetobacter* species and *Proteus mirabilis*. Distribution of these ESBL positive isolates was highest among urinary isolates, accounting for 84/179, (46.92%) of all the isolates recovered; and *Escherichia coli*s the most common organism producing ESBL (55 out of the 84 isolates).In pus samples, the percentage of ESBL positive isolates was 29/83 (34.93%) with *Klebsiella* species being the most common organism producing ESBL's (12/83). ESBL producing organisms were 20 out of the 62 sputum samples, with *Klebsiella* species being the foremost (12/62).

Figure 4
shows the sensitivity pattern of ESBL producing organisms. (Figure – 4 A & 4 B)

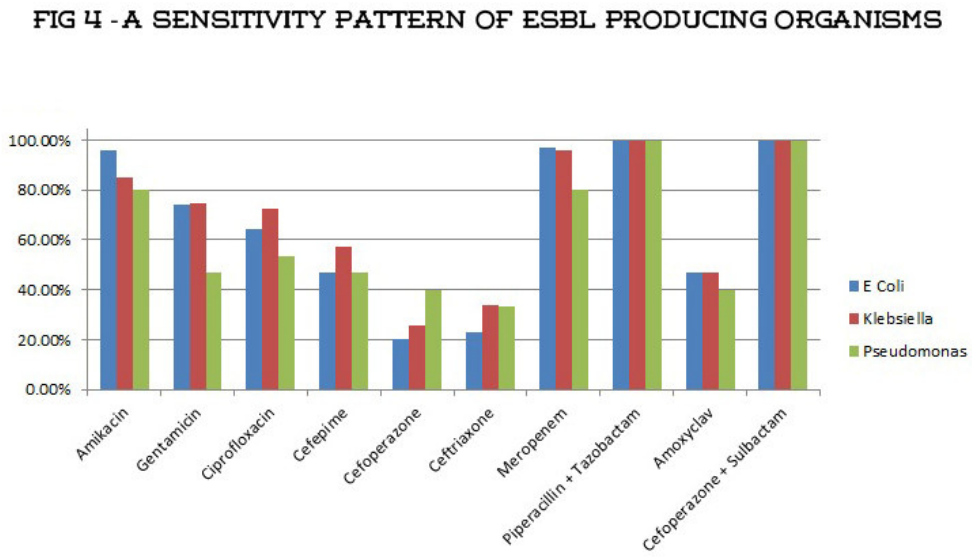


FIG 4 - B SENSITIVITY PATTERN OF ESBL PRODUCING ORGANISMS

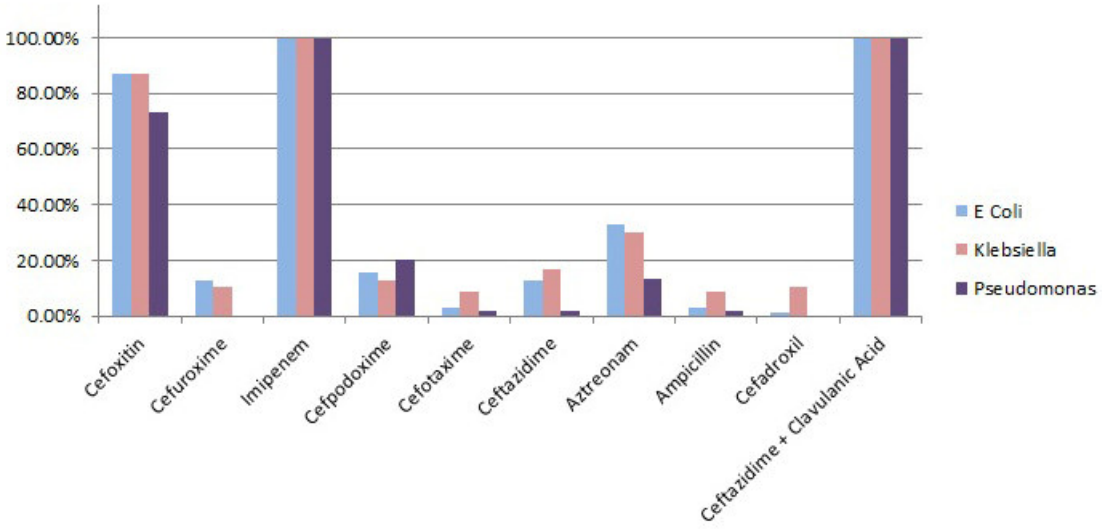


Table 3 shows the resistance pattern of ESBL and non-ESBL producing pathogens and their statistical significance.

Table 3
Resistance pattern of ESBL and Non ESBL producing pathogens and their statistical significance

Antibiotic	NESBL(R) 224	ESBL(R) 137	Significance/Non Significance
Amikacin	15	16	NS
Gentamicin	76	40	NS
Ciprofloxacin	113	47	$p < 0.01$
Cefepime	97	65	$p < 0.01$
Cefoperazone	83	103	$p < 0.001$
Ceftriaxone	57	98	$p < 0.001$
Cefadroxil	123	131	$p < 0.001$
Ceftazidime	111	118	$p < 0.001$
Cefotaxime	119	127	$p < 0.001$
Cefoxitin	71	21	$p < 0.001$
Aztreonam	78	110	$p < 0.001$
Cefuroxime	57	123	$p < 0.001$
Cefpodoxime	77	117	$p < 0.001$
Ampicillin	76	127	$p < 0.001$

DISCUSSION

Beta-lactam antimicrobial agents are the most commonly used antibiotics in the treatment of bacterial infections. Production of beta-lactamase is the most common mechanism of bacterial resistance. These enzymes are numerous and mutate continuously in response to the heavy pressure of a variety of beta-lactams leading to the development of Extended-spectrum Beta-lactamases. The genes encoding for ESBL's are often located on self-transmissible or mobilizable broad host range plasmids.[17] Resistant conjugative plasmids often code for resistant determinants to other antibiotics.[13]

The incidence of ESBL producing strains among clinical isolates has been steadily increasing over the past few years resulting in major setbacks in clinical therapeutics. Initially restricted to hospital acquired nosocomial infections, they have disseminated to involve people attending out-patient clinics also. Major outbreaks involving ESBL strain have been reported the world over, thus making them global emerging pathogens.[18] Detection of isolates producing ESBL is a challenge for the microbiology lab because these ESBL producing gram negative bacteria appear susceptible in-vitro to certain beta-lactam antimicrobial agents, yet result in clinical treatment failure. The routine antibiotic susceptibility testing is not capable of detecting ESBL producers without modification. Specific ESBL screening and confirmation tests should be conducted, otherwise, ESBL producers are likely to be reported falsely susceptible to the Cephalosporins leading to inappropriate antibiotic therapy. The present study proves that either the phenotypic confirmatory test or the E-test are equally effective and can be employed for the confirmation of ESBL production. In a resource limited laboratory, where E-test availability is difficult, detection of ESBL production can be carried out as a routine by phenotypic confirmatory combination disc diffusion test as it is simple and cost-effective. The bacteria causing UTI were the highest ESBL producers. This could be due to injudicious use of antibiotics in patients suffering from recurrent UTI infections and persistent use of indwelling Foley's catheters in patients within intensive care units. In the present study, 100% sensitivity to Imipernem was observed as also in other studies conducted by other researchers.[19,20] This finding advocates the usage of Carbapenem antibiotics as a therapeutic alternative in the wake of increasing resistance rates observed with conventional beta-lactam and non-beta-lactam antibiotics.[14]Amikacin was also found to be highly effective, with very few strains being resistant to it. Therefore, Carbapenems and Aminoglycosides can be therapeutically viable alternatives to the third generation Cephalasporins for the treatment of serious infections due to ESBL producing gram negative bacteria. Among the ESBL producing isolates, multidrug resistance was seen in 127 (92.7%) as against 94 (41.9%) non-ESBL isolates, the difference being statistically significant ($p< 0.01$). Mechanisms of co-resistance could possibly be explained as co-transmission of ESBL and resistance to other antimicrobials within the same conjugative plasmids.[21] Monitoring and judicious use of Extended-spectrum Cephalasporins, periodic surveillance of antibiotic resistance patterns and efforts to encourage empirical antibiotic therapy would contribute in alleviating some of the problems associated with ESBL's.

CONCLUSION

Almost all the ESBL producing organisms were multidrug resistant. Therefore, it is crucial to implement a revised strategy for empirical therapy, appropriate usage of Extended-spectrum Cephalosporins and regular assessment of antibiotic resistance pattern to control the spread of ESBL producing organisms in both community and hospital environments. ESBL production should be tested by the conventional methods and should be reported along with routine antibiotic susceptibility testing in every microbiology lab[22]. From the present study, we can conclude that detection of ESBL's should be carried out as a routine by the Phenotypic confirmatory combination disc diffusion test as it is simple, cost effective and user friendly.

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