

ORIGINAL RESEARCH ARTICLE

Screening of ESBL (Extended Spectrum of β - Lactamases) and Amp C β - Lactamases Producers among Pathogens Causing Urinary Tract Infections

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ABSTRACT

ESBL (Extended Spectrum of β - Lactamase) continue to be major problem in clinical world and exhibit co-resistance to many other classes of antibiotic resulting in limitation of therapeutic option. The purpose of the study was to simultaneously screen for ESBL and Amp-C β - lactamase in Gram negative isolates from hospital. It is a plasmid mediated enzymes first isolated in Europe in 1980, most commonly found in *Klebsiella* followed by *Escherichia coli*. These enzymes are capable of hydrolysing broad spectrum Cephalosporins and Monobactams, but inactive against Cephamycins and Imipenem. ESBL have serine at their active site and attack amide bond in the β lactam ring of antibiotic causing their hydrolysis. A total of 60 clinical isolates comprising *Escherichia coli* (n=44,73), *Klebsiella* sp. (n=10,17), *Proteus* sp. (n=3) and *Pseudomonas* sp. (n=3) were recovered from urine sample over a period of four months (January to April 11). Antibiogram profiles of these isolates were determined along with screening for ESBL and Amp-C β - lactamase production by phenotypic detection method as recommended by the Clinical Laboratory Standard Institute (CLSI). Two tests were performed for each, combination disk and synergy for ESBL and three dimensional and disk antagonism test for Amp-C β - lactamase detection. Out of the 60 isolates, 42 (70%) isolates were found to be ESBL producer. From these, 34 (81%) *Escherichia coli*, 6 (14%) *Klebsiella* sp., 2 (5%) *Proteus* sp. Amp-C was found to be 20 (43%) isolates, of these 17 (85%) *Escherichia coli* and 3 (15%) *Pseudomonas* sp. Then, the Prevalence of ESBL producer was found to be 70% (42/60). All the isolates were sensitive to Imipenem.

Key words: ESBL, Amp-C β – Lactamases, Cephalosporins, Imipenem and Uropathogens.

1. INTRODUCTION

Extended spectrum beta lactamases (ESBL) are enzymes which break down many common antibiotics and make them ineffective in fighting infection caused by bacteria. It has a molecular mass of 29 kDa. Beta lactamases production by several Gram negative and Gram positive organism is perhaps the most important single mechanism of resistant to Penicillin and Cephalosporins^[1]. In the past, it was believed that Cephalosporins were relatively immune to attack by β -lactamase. It was surprising to find cephalosporin resistant Gram negative bacteria among the clinical isolates due to the production of ESBL^[2]. Resistant bacteria are emerging worldwide causing life threatening infection in community and hospital settings^[3]. Antibiotic resistance in uropathogens is increasing worldwide in both outpatients as well as in hospitalised patients. It varies according to

geographical locales and is directly proportional to the use and misuse of antibiotics. Understanding the impact of drug resistance is of critical importance as the changing rate of antibiotic resistance has a large impact on the empirical therapy of urinary tract infections^[4].

Urinary tract infection (UTI) is the second most common infection. Worldwide about 150 million people are diagnosed with UTI each year, costing more than 6 billion US dollars. Among both out patients and inpatients, *Escherichia coli* is the primary clinical isolate accounting for 75% to 95 % of uncomplicated UTI isolate. *Klebsiella*, *Proteus*, *Pseudomonas*, *Enterococcus* and *Enterobacter* sp. are less commonly isolated from outpatients^[5]. UTI may involve only the lower urinary tract or may involve both the upper and lower tract. The term cystitis has been used to describe lower UTI, which is characterized by syndrome involving dysuria, urgency and

occasionally supra pubic tenderness. However, the present symptoms of lower urinary tract without upper urinary tract symptoms does not exclude upper urinary tract infection, which is also often present [6].

The various mechanism of drug resistance in Gram negative bacilli includes ESBL production, AmpC β lactamase production, efflux mechanisms and porin deficiency [7]. The mechanism of resistance to third generation Cephalosporins is due to production of ESBL and AmpC β lactamase are most common [8]. The resistant pattern of uropathogens has not been extensively studied in Indian sub-continent [9].

ESBL are distinguished into more than 30 types based on their physical properties and all are inhibited by Clavulanate, Sulbactam and Tazobactam a property which has been used to detect them in laboratory [10]. The ESBL enzymes are plasmid - mediated enzymes capable of hydrolyzing and inactivating a wide variety of β lactams, including third generation Cephalosporins, Penicillins and Aztreonam, where Amp C β -lactamase was both plasmid and chromosome encoded enzyme [11]. Amp-C class β -lactamase are cephalosporinase that are poorly inhibited by Clavulanic acid. It can be differentiated from other ESBLs by their ability to hydrolyse Cephameycins (Cefmetazole, Cefotetan, Cefoxitin) as well as other extended spectrum Cephalosporins [12]. But the ESBL enzymes have no detectable activity against Cephameycins and Imipenem. Because of their greatly extended substrate range these enzymes were called ESBLs [13].

The first ESBL isolates were discovered in Europe in 1983 and US in 1998. In India it is detected in many hospitals. The major ESBL producing strains are *K. pneumoniae*, *K. oxytoca*, *E. coli* and other organisms reported to harbour ESBL include *Enterobacteriaceae sp*, *Salmonella*, *Morgenella morgani*, *Proteus mirabilis*, *Serratia marcescens* and *Pseudomonas aeruginosa* [11]. With this background following, the present study was undertaken to study the incidence of ESBL producers and Amp-C β lactamase pathogens causing UTI and their susceptibility pattern to other antibiotics.

2. MATERIALS AND METHODS

2.1. Specimen Collection and Transport

Urine samples were collected from both the sexes based on the patients of irrespective age group having urinary tract infection in Meenakshi Medical Hospital, Kanchipuram from January to

April of 2011. Samples were obtained by using sterile container and transported to the laboratory immediately.

2.2. Isolation and Identification of the Bacterial Isolates

The collected specimens were utilized for the following experiments. Colony morphology, Gram staining, Motility and Biochemical characterisation such as Indole, Citrate, TSI, Mannitol motility, Urease test were carried out as per the methods explained by CLSI to identify the bacterial isolates from the urine sample. The isolates were stored in Brain Heart infusion broth at -20°C until simultaneously tested by the various methods discussed below. Prior to testing, each isolate was sub cultured on nutrient agar to ensure purity.

2.3. Antimicrobial Susceptibility Testing

The sensitivity of the isolates to third generation cephalosporin's (3GC) viz., Ceftazidime, Cefotaxime, Ceftioxone each 30 μ g/disk and to the other antibiotics such as Amikacin (30 μ g), Ampicillin (10 μ g), Gentamycin (10 μ g), Co-Trimoxazole (25 μ g), Tetracycline (30 μ g), Imipenem (30 μ g), Cefoxitin (30 μ g), Aztreonam (30 μ g), Chloramphenicol (30 μ g) was determined by the Kirby Baure's disk diffusion method [13]. Plates were inoculated with a bacterial suspension matched with 0.5 McFarland standards. Standard antibiotic disk were aseptically placed at reasonable equidistance on the inoculated Mueller Hinton agar plates. The plates were then incubated at 37 °C for 24 hours. The diameter of zone of inhibition produced by each isolates were measured, recorded and the isolates were classified as resistant, intermediate and sensitive based on the standard interpretative chart updated according to the Clinical and Laboratory Standard Institute (CLSI) recommendations [14]. *Escherichia coli* ATCC 25922 strain was used for the quality control. Isolates with resistance or with decreased susceptibility to any of the 3GC were selected for further study.

2.4. Detection of ESBL - Double Disk Diffusion Synergy Test (DDST)

In the DDST, synergy was determined between a disk of Augmentin (20 μ g Amoxicillin and 10 μ g Clavulanic acid) and a 30 μ g disk of each 3GC test antibiotic placed at a distance of 20-25mm apart on a culture of the resistant isolate under test on Mueller Hinton Agar (MHA) (16). The test organism was considered to produce ESBL, if the zone size around the test antibiotic disk increased towards the Augmentin disk. This increase occurs

because the Clavulanic acid present in the Augmentin disk inactivates the ESBL produced by the test organism. Since 52 isolates were found to be resistant to at least one of the 3GC test antibiotics they were tested for ESBL production by DDST. In the same test synergy was also determined between a disk of Ceftazidime + Clavulanic acid and all 3GC test antibiotic^[15].

2.5. Double Disk Approximation Method/Combination Disk Method

The combination disk method was used to confirm the presence of ESBL on all the isolated isolates by placing a disk of Ceftazidime (30 μ g) alone and Ceftazidime (30 μ g) in combination with Clavulanic acid (10 μ g) on MHA plate. The disks were placed at least 20mm apart from each other on MHA plate. If the zone diameter around Ceftazidime +Clavulanic acid disk is >5mm larger than that around Ceftazidime disk alone will be considered as ESBL producer. Likewise, the test is repeated for disk of Cephalexin, Cefepime, Cefepirome alone and with their combination^[16].

2.6. Detection of Amp-C β - Lactamases - Disk antagonism test

The Cephalosporin such as Ceftazidime, Ceftriaxone, Cephalexin and Cefepime were placed around the Cefoxitin on MHA plate inoculated with the test organism. The plates were incubated at 37°C for 24 hours. Blunting of the cephalosporin disk adjacent to Cefoxitin disk was interpreted as a positive test^[17].

2.7. Three Dimensional Extract Test

50 μ l of 0.5 McFarland adjusted bacterial suspension was inoculated into 12 ml of MHB and incubated for 4 hours. The cells were concentrated by centrifugation and the crude enzyme was prepared by sonicating the pellets (in sonicator for 15 seconds with 10 seconds cooling in between sonications). MHA plates were inoculated with *Escherichia coli* ATCC25922. 30 μ g Cefoxitin disk were placed in the center. With a sterile scalpel blade a slit beginning at 5 mm from the edge of the Cefoxitin disk was cut in the agar in outward radial direction. The enzyme preparation was dispensed in the slit beginning near the disk and moving outward radially. The plates were incubated at 37°C for 24 hours. Enhanced growth of surface organism at the point where the slit intersects the zone of inhibition due to Cefoxitin

was considered as a positive test and evidence for the presence of Amp-C β -lactamases. The test was repeated with enzyme extract with a 5 μ g Cloxacillin disk added to the extract and incubated at 37°C for 30 minutes. Three different kinds of result were recorded. The isolate showing clear distortion of zone of inhibition of Cefoxitin were taken as Amp-C producers. The isolate with no distortion were taken as Amp-C non producers and isolate showing minimal distortion were taken as intermediate strains. Inhibition of zone distortion when Cloxacillin disc in enzyme extract were used confirmed Amp-C producers^[18].

2.8. E-test

10 μ l of bacterial suspensions matching 0.5 McFarland turbidity standards were plated on MHA followed by application of E-test strips. The plates were incubated at 37°C for 24 hours. MIC was interpreted as the zone of inhibition corresponding to concentration gradient on E-test strips^[19].

3. RESULTS

3.1. Isolation and Identification of Bacterial Isolates

Of the 60 strains isolated from Urinary Tract Infection expected patients specimens, 44 were *Escherichia coli* 73% (44/60) followed by *Klebsiella pneumoniae* 17% (10/60), *Pseudomonas aeruginosa* 5% (3/60) and *Proteus* sp. 5% (3/60) were obtained.

3.2. Susceptibility Testing by Disk Diffusion Method

A total of 60 isolates were tested by Kirby-Bauer disk diffusion method, of which 54 (90%) isolates were resistant to Ampicillin, 3 (5%) isolates were resistant to Amikacin, 10 (16.67%) isolates were resistant to Chloramphenicol, 49 (81.67%) isolates were resistant to Co-trimoxazole, 52 (86.67%) isolates were resistant to Carbenicillin, 44 (73.33%) isolates were resistant to ciprofloxacin, 10 (16.67%) isolates were resistant to Nitrofurantoin, 44 (73.33%) isolates were resistant to Norfloxacin, 45 (75%) isolates were resistant to Tetracycline, 10 (16.67%) isolates were resistant to Piperacillin or Tazobactam. 47 (78.33%) isolates were resistant to Cefoxitin, 49 (81.66%) isolates were resistant to Aztreonam. All the isolates were sensitive to Imipenem (Table 1).

Table: 1. Percentage of Antimicrobial Resistance in clinical isolates

S. No	Antimicrobials	Bacterial Isolates							
		<i>E. coli</i> n=44		<i>Klebsiella</i> n=10		<i>Pseudomonas</i> n=3		<i>Proteus</i> n=3	
		No.	%	No.	%	No.	%	No.	%
1	Ampicillin	39	88	9	90	3	100	3	100

2	Amikacin	2	4	0	0	0	0	1	33
3	Chloramphenicol	7	15	1	10	0	0	2	66
4	Co-trimoxazole	35	79	8	80	3	100	3	100
5	Carbenicillin	39	88	9	90	1	3	3	100
6	Ciprofloxacin	38	86	5	50	0	0	1	33
7	Imipenem	0	0	0	0	0	0	0	0
8	Nitrofurantoin	3	6	2	20	3	100	2	66
9	Norfloxacin	37	84	5	50	0	0	2	66
10	Tetracycline	33	75	4	40	3	100	3	100
11	Piperacillin/tazobactam	8	18	2	20	0	0	0	0
12	Aztreonam	37	84	7	70	2	66	3	100
13	Cefoxitin	37	84	6	60	3	300	1	33

3.3. Test for ESBLs detection - Double Disk Diffusion Synergy Test (DDST)

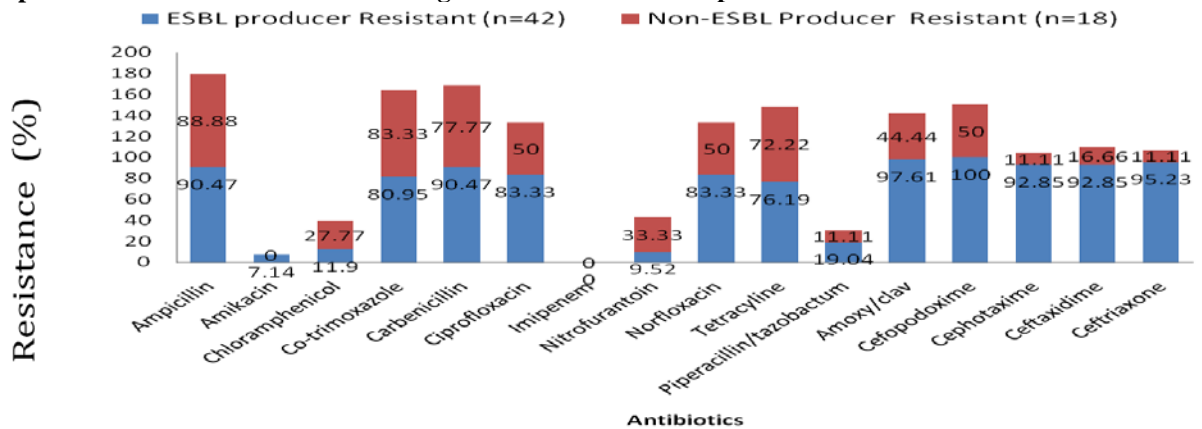
A total of 60 isolates were tested using amoxy/clav (augmentin) disk, of which 70% (42/60) isolates were positive for ESBL production. Of the 42 isolates, 81% (34/42) were *Escherichia coli*, 17% (7/42) *Klebsiella pneumoniae*, 2% (1/42) *Proteus* sp. were found to be ESBL producer. When the isolates were tested using Ceftazidime + Clavulanic acid (CAC), the percentage of ESBL producers were 68.33% (41/60) isolates were positive for ESBL production. Of the 41 isolates, 80% (33/41)

Escherichia coli, 15% (6/41) *Klebsiella pneumoniae*, 5% (2/41) *Proteus* sp. were found to be ESBL producer. In both of these methods, none of the *Pseudomonas* sp. found to be an ESBL producer (Fig 1 & Graph 1).

3.4. Double disk approximation/combination disk method

A total of 70% (42/60) isolates were positive for ESBL production. Of the 42 isolates, 81% (34/42) *Escherichia coli*, 14% (6/42) *Klebsiella pneumoniae*, 5% (2/42) *Proteus* sp. were ESBL producer (Fig 2 & Graph 2).

Fig 1: Comparison of antibiotic resistance among ESBL and non-ESBL producer

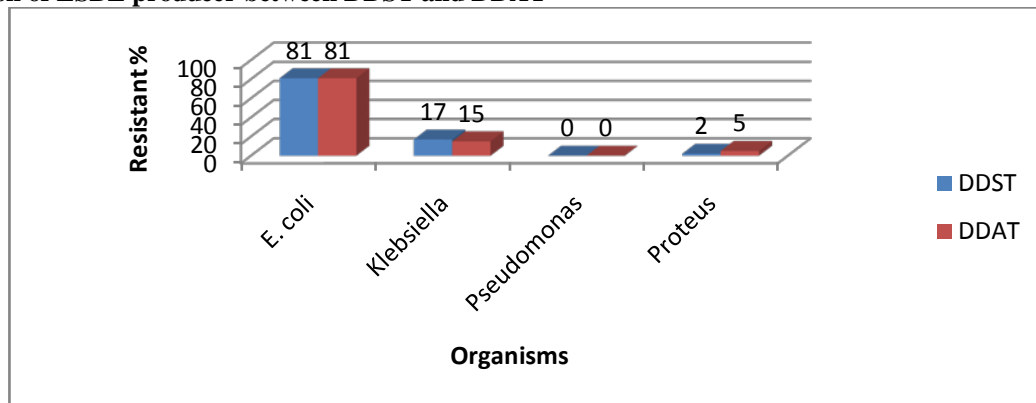


3.5. Test for Amp C β -lactamases - Disk antagonism test

Of the 47 Cefoxitin resistant strains tested by the disc antagonism test, 20 (43%) isolates were

positive for inducible β -lactamases. It was further confirmed by three dimensional extract test (Fig 3).

Fig 2: Comparison of ESBL producer between DDST and DDAT



DDST: Double Disk Synergy Test; DDAT: Double Disk Approximation Test

Fig 1: Double Disk Synergy Test

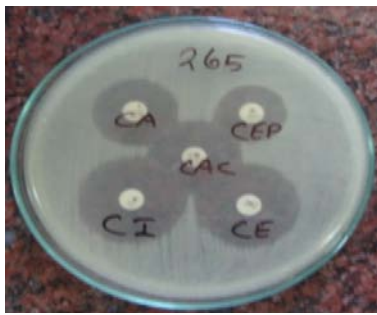


Fig 2: Double Disk Approximation Test

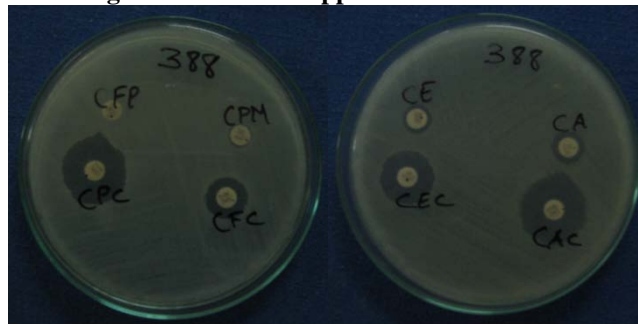
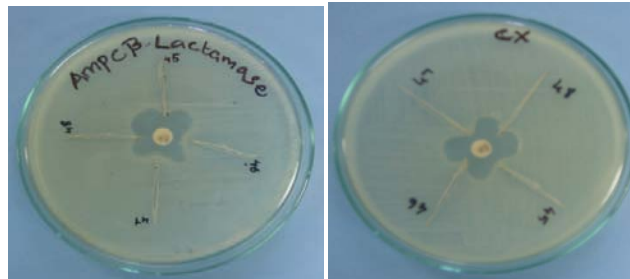


Fig: 3. AmpC β - lactamases



3.6. Three Dimensional Extract Test

A total of 47 Cefoxitin resistant isolates were tested, among that 20 (43%) isolates produce Amp-C β -lactamase and it was further confirmed

by using 5 μ g Cloxacillin antibiotic disk of these, 85% (17/20) *Escherichia coli* and 15% (3/20) *Pseudomonas* sp. (Table 2).

Table 2: Comparison of ESBL and AmpC β -lactamase producer among clinical isolates:

Organism	ESBL producer (n=42)	AmpC producer (n=20)
<i>E. coli</i>	34 (81%)	17(85%)
<i>Klebsiella</i> sp.	6 (14%)	-
<i>Pseudomonas</i> sp.	-	3 (15%)
<i>Proteus</i> sp.	2 (5%)	-

3.7. E-TEST(Minimum Concentration)

A total of 20 isolates were tested for Ceftazidime, Cephotoxime, Ceftriaxone, Cefepime E strip of these, 25% (5/20) Intermediate and 75% (15/20) were found to be resistant for Ceftazidime. 35%

Inhibitory

(7/20) Intermediate and 65% (13/20) were resistant for Cephotoxime, 40% (8/20) sensitive, 20% (4/20) intermediate and 40% (8/20) Resistant for Ceftriaxone. 45% (9/20) sensitive, 30% (6/20) intermediate and 25% (5/20) sensitive for Cefepime (Table 3 & Fig 4).

Table 3: E TEST

Antibiotic	No. of Antibiotic Resistance in conc. of μ g/ml											
	8	%	16	%	32	%	64	%	128	%	256	%
Ceftazidime	-	-	5	25	6	30	4	20	-	-	5	25
Cephotoxime	-	-	2	10	5	25	6	30	2	10	5	25
Ceftriaxone	8	40	1	5	3	15	4	20	1	5	3	5
Cefepime	9	45	4	20	2	10	1	5	1	5	3	5

Fig 4: E - Test



4. DISCUSSION

In the present study, the prevalence of ESBL producer was found to be 70% (42 out of 60)

among *Escherichia coli*, *Klebsiella*, *Pseudomonas*, *Proteus* isolates collected over four months. The overall prevalence of ESBL

producers was found to vary greatly in different geographical areas and in different institutes. In US, the occurrence of ESBLs in Enterobacteriaceae ranges from 0 to 25%. In Russia, Poland and Turkey is 39-47% and the national average is 3%. In India, the prevalence rate is 28 -84% [20].

For a numerous reason, the detection of ESBL and AmpC β lactamses producing strains is significant importance for all major hospitals worldwide. First, the strains are more prevalent due to this very difficult to detection by the clinical methods [21]. Second, treatment of the ESBL infection is very difficult because of the resistant to extended spectrum Cephalosporins. It makes serious threat to current β lactam therapy. Third, institutional outbreaks are increasing because of selective pressure due to the heavy use of extended spectrum Cephalosporins [3]. The optimum substrates profile various from one ESBL enzyme to another. For this reason, susceptibility range with only one extended spectrum cephalosporin cannot predict resistance to other extended spectrum Cephalosporins [22].

Almost all the ESBL positive isolates were found to be resistant to Ampicillin, Ciprofloxacin, Norfloxacin, Tetracycline and sensitive to Imipenem, Chloramphenicol and Nitrofurantoin. It suggest that use of Carbapenem antibiotics as the therapeutic alternative to β -lactam antibiotics as indicated in many previous studies. AmpC β lactamase are more susceptible to Tazobactam as compared to Clavulanic acid and sensitive to Cefepime. Both ESBL and non ESBL producer showed high level of resistance to Cefepime (fourth generation Cephalosporin).

5. CONCLUSION

The prevalence of ESBL producer was 70% at Meenakshi Medical Hospital. Routine detection of ESBL producing microorganism is required to be done on each laboratory by the standard detection method, so as to control the spread of these infection and also to instruct proper therapeutic strategies. Phenotypic confirmatory test using combination disk is simple and cost effective for the detection of ESBL. The control measures include judicious use of antibiotics, strict hand hygienic protocols and implementation of appropriate infection control measures in the hospital, especially while treating high risk patients.

Monitoring and judicious usage of extended spectrum cephalosporins, periodic surveillance of antibiotic resistance pattern and efforts to decrease

empirical antibiotic therapy would go a long way in addressing some problems associated with ESBLs.

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