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ORIGINAL ARTICLE

Prevalence of Extended Spectrum Beta-Lactamases (ESBL) Producers among Gram-Negative Bacilli in Urinary Tract Infection (UTI)

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ABSTRACT
Urinary tract infections are one of the most common bacterial infections in humans. It is also one of the common specimens sent to the laboratory. Worldwide, the data show that there is an increasing resistance among the organisms which cause UTI.Extended Spectrum β -Lactameses Producing Organisms (ESBLs) are increasing in number and causing more severe infections because of their continuous mutation and multidrug resistance property with limited therapeutic option. Present study was undertaken to detect the prevalence of the ESBLs producing bacteria in UTI so as to provide a base line data in treating them & prevent unnecessary use of antibiotics. A total of 422 samples collected from various departments during the period of October 2018 to November 2019 were processed in various culture medium and ESBL isolates were detected using DDST and E test. Escherichia coli was the common isolate. Escherichia coli was the most common ESBL producer(67.5%). E test was more sensitive than DDST in detecting ESBL. ESBL isolates are more susceptible to Meropenem,Nitrofurantoin, and Amikacin. DDST can also be adopted as a method ofESBL detection.: It is essential to report ESBL production along with routine sensitivity reporting, which will help the clinician in prescribing the proper antibiotics.

Keywords: *Urinary Tract Infection, (UTI), ESBL, AST, DDST, E Test, β-lactamase, TEM, SHV, CTX-M*

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INTRODUCTION

Urinary tract infections (UTIs) are one of the most prevalent bacterial infections in the developing countries. Among the hospital acquired infections, UTI contributes to 35-40% of them. They also cause morbidity and increased mortality in hospitalized patients. It has a spectrum of clinical entities with severity ranging from asymptomatic infection to acute pyelonephritis with sepsis.

Antimicrobial resistance is a growing threat worldwide. Resistance mechanisms are found for almost every class of antibiotic agents. The production of beta lactamases is the important mechanism of resistance in Gram negative bacteria. The production of extended-spectrum β-lactamases (ESBL) is an important mechanism of resistance to third-generation cephalosporins which are widely used in urinary tract infections because of lesser nephrotoxicity effects. During last few decades ESBL producing Gram-negative organisms have been a major problem in many settings.

Extended Spectrum Betalactamases

Extended spectrum beta lactamases (ESBL) are enzymes capable of conferring bacterial resistance to the penicillins, first, second and third generation cephalosporins and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics. They are inhibited by beta lactamase inhibitors such as clavulanic acid, sulbactum and tazobactum. They are thought to have evolved by mutation of the TEM and SHV genes. Organisms responsible for UTIs such as *E.coli* and *Klebsiella* produce these enzymes. These enzymes are plasmid borne and confer multi drug resistance, difficulty in treatment and increased morbidity and mortality. Detection of ESBLs in urine samples are important because they represent epidemiological marker of colonization. Disc diffusion methods have been proposed by CLSI for the

detection of ESBLs in organisms such as *Klebsiella*, *Escherichia coli* and *Proteus*. The reduction in the zone diameters of antibiotics such as cefpodoxime, ceftriaxone, ceftazidime, cefotaxime or aztreonam indicates ESBL production. Testing with more than one drug increases the sensitivity of detection. Phenotypic confirmatory methods, such as double disc synergy test, combined disk method and E test (epsilometry methods) are available which are based on in vitro inhibition of ESBLs by betalactamase inhibitors, Genotypic detection methods are useful in distinguishing between specific enzymes (TEM, SHV, CTX-M) responsible for ESBL production and for epidemiological purposes.

As the specimen load (urine specimen) received in the laboratory have been increasing and the multidrug resistant organisms causing serious infections are on the rise, this study has-been taken up. The spectrum of organisms causing UTI in our hospital with their antibiotic susceptibility pattern has been evaluated. In this study phenotypic methods such as double disc synergy tests and E tests are used in the detection of ESBLs among Gram negative isolates.

Extended spectrum beta lactamases are enzymes capable of hydrolyzing third and fourth generation cephalosporins andmonobactams, but inactive against cephamycins and imipenem. They are inhibited by betalactam inhibitors such as clavulanicacid, Sulbactam and tazobactum. The ESBLs have amino acid serine at their active site and they attack amide bond in the lactamring of these beta lactam antibiotics, causing subsequent lysis. They belong to Bush-Jacoby-Medeiros group 2be, 2d and Ambler class A.The type of ESBLs is TEM, SHV, CTX-M, OXA, Amp C but majority are derived from TEM or SHV enzymes. These enzymes are commonly found in *E.coli* and *Klebsiella pneumoniae*. Sometimes more than one gene may be present in the same organism.

Prevalence

Prevalence of ESBLs vary in different countries and indifferent areas. In India the prevalence varies from 8 to 80%. The prevalence of TEM and SHV are 56% and 60% respectively.

Gene Encoding ESBL

TEM

TEM-1 enzyme was first reported from *E.coli* in 1965 and now it is the commonest betalactamase found in *Enterobacteriaceae.* The name TEM is derived from Temoniera, a patient from Greece from whom the strain was isolated. Based on the combination of different amino acid changes, currently 195 TEM type enzymes have been found.

SHV

The name SHV denotes for sulfhydryl variable. About 68%of amino acid is shared with TEM-1. SHV -1 is most commonly found in *Klebsiella pneumoniae*. ESBLs in this family have amino acid changes at 238 positions. There are about 60 SHV variants are known. SHV-5 was found to be responsible for outbreaks of nosocomial infections in several countries.

CTX-M

CTX-M beta lactamases were first isolated from Munich in Japan in 1986. *E.coli* producing CTX-M have emerged as an important cause of community onset urinary tract infections. These enzymes have greater activity against cefotaxime. Change at 236positions enhances resistance to cefotaxime and change at 102position enhances resistance to ceftazidime. There are about 80CTX-M enzymes which are divided into 5 clusters on the basis of amino acid sequence. CTX-M producing *Escherichia coli* are emerging as an important pathogen causing community onset and hospital acquired infections.

In India CTX-M -15 beta lactamase, is the most prevalent. The bowel provides a rich environment for genetic exchange between commensal *Enterobacteriaceae*. Conditions such as overcrowding, poor sanitation and inappropriate use of antibiotics contribute to the widespread prevalence of CTX-M- 15 beta lactamases. Some organisms may harbour more than one type of ESBLs which may alter the antibiotic resistance phenotype.

Organisms Producing ESBLs

Escherichia coli,*Klebsiella pneumonia*, *Proteus spp*, *Enterobacter*,*Citrobacter*and *Pseudomonas.*

Risk Factors Associated with ESBL Production

- Prolonged stay in intensive care units.
- Poor nutritional status.
- Severe illness.
- High rate use of ceftazidime and other third generation cephalosporins.
- Long term use of antibiotics.
- Use of central venous lines, urinary catheters, endotrachealtubes,arterial lines, nasogastric tubes.
- Haemodialysis.
- Administration of total parenteral nutrition.

Laboratory Methods of Identification of Extended Spectrum Beta Lactamases

- Phenotypic methods
- Genotypic methods

Phenotypic methods are commonly performed by the laboratories because they are easy to perform and cost effective also. These tests depend on detecting synergy between clavulanic acid and the indicator cephalosporins.

Phenotypic Confirmatory Tests

- **Double-Disk Synergy Test**

This method of phenotypic confirmation was proposed in 1980 explained by Jarlier et al. Organism to be tested is placed on the Mueller-Hinton agar. An antibiotic disc containing one of the beta lactam antibiotics is placed 30-15 mm apart (centre to centre) from amoxicillin-clavulanic acid disc. Following overnight incubation in air at 37^{o}C, the discs are read. Enhancement of zone of inhibition of the beta lactam antibiotic caused by synergy of clavulanate indicates a positive result. Sensitivity is increased by reducing the distance between the discs to 20 mm. Addition of clavulanate to the Muller-Hinton agar increases the sensitivity.

Disc Strength: Cephalosporin (3rd generation)- 30µg/disc.

Coamoxyclav: 20µg amoxicillin+10µg clavulanic acid.

Genotypic Detection

Many of the research laboratories use genotypic methods for the identification of the specific genes responsible for the production of ESBLs. They also have the ability to detect ESBLs which are not detected by phenotypic methods. They also have the advantage that they can be done directly on clinical specimens without culturing bacteria, with subsequent reduction of the turnaround time. The molecular methods in use are DNA probes, Polymerase chain reaction. Restriction fragment length polymorphism, Single strand conformational polymorphism and Ligase chain reaction. Most of them are specific for gene family and detects TEM and SHV variants. Among these nucleotide sequencing is a gold standard, since it is capable of detecting all variants.

Screening Tests

The current Clinical and laboratory standard institute guidelines for detection of extended spectrum beta lactamases in *Klebsiella spp*. and *E. coli* includes an initial screening test with any two of the following beta lactam antibiotics : cefpodoxime, ceftazidime, aztreonam, cefotaxime or ceftriaxone. The use of more than one of the five beta lactam antibiotics will improve the sensitivity of detection. Cefpodoxime and ceftazidime show the highest sensitivity for ESBL detection. There is also a possibility of missing some strains if the inoculum is very low.

Treatment

The plasmid having the genes encoding for ESBLs also carry genes which encodes resistance to aminoglycosides and cotrimoxazole. Porin loss was observed in strains producing ESBLs whichleads to quinolone resistance. But for urinary tract infections quinolones can be prescribed if there is in vitro susceptibility.β lactam/ β lactamase inhibitor combinations are active against organisms possessing a single ESBL only. Additionally ESBL producing organisms may harbour parent enzymes (TEM-1 orSHV-1).Hyper production of these enzymes and porin loss maycontribute to reduction in the activity of beta lactamase inhibitors. Hence β lactam /β lactamase inhibitor combinations are not regarded as first line therapy for serious infections with ESBL producing organisms. But for urinary tract infections it can be a second line therapy.

Fosfomycin and Nitrofurantoin are the treatment of choice, for uncomplicated cystitis. Cefepime resistance is seen in strains producing CTX-M type ESBLs. Hence cefepime, if at all used for therapy should be used in high dosage. Carbapenems have the most consistent activity against ESBL producing organisms. Hence should be regarded as the drugs of choice for serious infections with ESBL producing organisms.

MATERIAL AND METHODS

ESBL Detection

The method recommended by Clinical Laboratories Standard Institute (CLSI) requires a two step approach of initially screening for ESBL production and then performing confirmatory tests on screen positive isolates.

Screening For Extended Spectrum Betalactamase

As per CLSI 2016 guidelines, the test isolates which showed an inhibition zone of ≤ 27mm for cefotaxime (CTX), ≤25mm for ceftriaxone(CTR) and ≤ 22mm for ceftazidime (CAZ) were considered as presumptive

ESBL producer. Only the organisms such as *Escherichia coli*, *Klebsiella* and *Proteus* were subjected to confirmatory test.

All these isolates were further tested for phenotypic confirmation for ESBL production by Double Disc Diffusion Method as per CLSI 2016 guidelines. A lawn culture was made on MHA plate with the test isolate, and then Cefotaxime, Ceftazidime discs with and without Clavulanic acid discs were placed. The plates were incubated at 37°C overnight. The isolates were considered as ESBL producer when zone of inhibition around the combination disc (CAZC/ CEC) is more than or equal to 5mm than the cephalosporin disc alone. The findings were recorded and noted.

Double Disc Synergy Test

Test organism was inoculated in peptone water and lawn culture was made on Mueller-Hinton agar as recommended for a standard disk diffusion susceptibility test. Discs containing 30µg of ceftazidime and 30µg cefotaxime were placed 15 mm apart (centre to centre) to the disc containing Amoxicillin/Clavulanic acid (20/10µg). Plates were incubated at 37^0C overnight. Enhancement of zone ofinhibition between the clavulanate discs and any one of the β-lactam antibiotic disc was interrupted as an indication for ESBLproduction.

E Test (Epsilometer Test)

EM079AIt is a phenotypic ESBL detection strip which is coated with mixture of three different cephalosporins with and without clavulanic acid on a single strip in a concentration gradient manner. The upper half has Ceftazidime, Cefotaxime and Cefepime +Clavulanic acid and Tazobactum with concentration gradient tapering downwards. The lower half is coated with Ceftazidime, Cefotaxime and Cefepime in a concentration gradient in reverse direction.

Procedure

- An inoculum was prepared from the pure culture of the test organism . It was standardized with that of the 0.5 McFarland standards.
- Mueller-Hinton agar was prepared in-house according to manufacturer's instructions.
- A sterile cotton swab was dipped into the standardized inoculum and was rolled to express excess fluid.
- Entire agar surface was streaked with the swab three times turning the plate at 60^0 angle between each streaking.
- Strip was removed from the container with an applicator and was placed on the agar plate.
- Plates were kept in the incubator and read on the next day.
- Values were read where the ellipse intersects the scale on the strip.

Interpretation

The following criteria were used for interpretation. It was interrupted as ESBL positive. When the ratio of the value obtained for (Mix/ Mix+) was greater than or equal to 8, or nozone was obtained for MIX and zone obtained in Mix +.It was interrupted as ESBL negative strain when the ratio of the value obtained for (Mix/ Mix+) was less than eight.

It was considered, non conclusive for ESBL when no zone of inhibition was obtained on either side. In such cases resistance maybe due to some other mechanisms other than ESBL production. Quality control of the strips was carried out by testing the strips with the standard ATCC cultures.

Positive control –*Klebsiella pneumonia* ATCC 700603

Negative control – *Escherichia coli* ATCC 25922

The information collected regarding all the selected cases were recorded in a Master Chart. Data analysis was done with the help of computer using Epidemiological Information Package (EPI 2002).

Figure1: AST pattern of ESBL Isolate

Figure2: Estrip with ESBL Positive Isolate

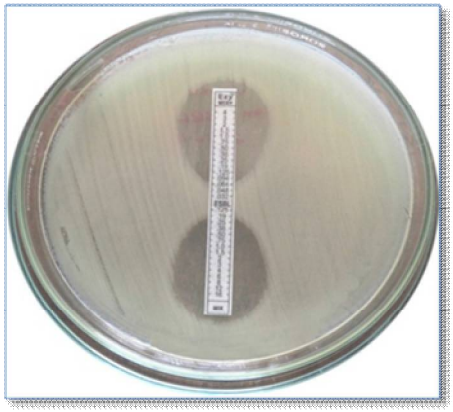

Figure 3: Estrip with ESBL Negative Isolate

Figure 4:DDST with ESBL Positive Isolate

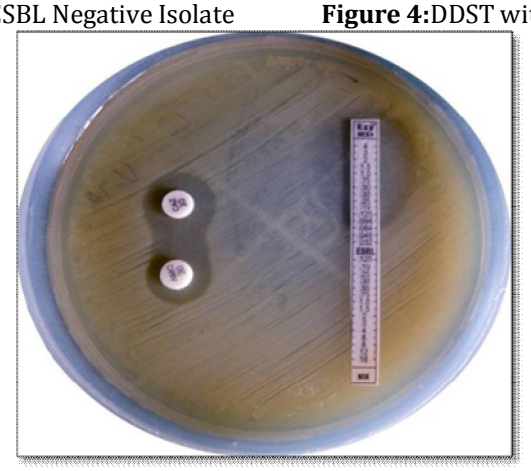

Figure6: Estrip and DDST with ESBL Positive Isolate

RESULT AND DISCUSSION

Table 1: Percentage of ESBL Organisms (n=67)

Name of the Microorganism	Total Number	ESBL Positivity	Percentage (%)
E. coli	77	52	67.5
Klebsiella pneumonia	15	10	66.7
Proteus sp.	9	5	55.6

All the Cefotaxime resistant isolates of *E. coli*, *Klebsiella* and *Proteus* were subjected to confirmatory tests by double disc synergy test and E tests. The results were shown in Table 1. Among the 67 ESBL positive isolates, *Escherichia coli* 52 (67.5%) was the predominant ESBL producing organism. The ESBL positiveisolates among *Klebsiella* and *Proteus* were 10 (66.7%) and 5(55.6%) respectively.

Table 2: Comparison of ESBL detection using the DDST and E Test

Microorganism Positive in ESBL Screening	Microorganisms Positive in Double Disc Synergy Test (DDST)	Microorganisms Positive in E test
E. coli (54)	48	52
Klebsiella (10)	10	10
Proteus (6)	5	5
Total (70)	**63 (90 %)**	**65 (95.7 %)**

The comparison of ESBL detection using double disc synergy test and E test was depicted in Table 2. Out of 70 isolates which were positive in screening tests, 63 were detected as ESBL producers from double disc synergy test and 67 were detected in E test. Out of these two tests E test detected maximum number of organisms (95.7%) compared to DDST (90%).

In our study, ESBL screening was done using Cefotaxime disc which was similar to the study done. In this, they have proved that all the ESBL producers were uniformly resistant to all three third generation

cephalosporins (Cefotaxime, Ceftriaxone and Ceftazidime). Confirmation was done by double disc synergy test and E test. Out of 70 isolates which were positive ESBL producers in screening test, 63(90%) were positive in DDST test and 67 (95.7%) were positive in E test. Two *Escherichia* strains and one *proteus* strain produced inconclusive results. This could be due to some other resistance mechanism other than ESBL production such as AmpC production. Our study shows that E test was more sensitive compared to double disc synergy test which was also proved. A study by showed that E test was 100% sensitive and 97.6% specific with 97.3% positive predictive value.

Table 3: Sex Wise Distribution of ESBL Positive Isolates (n=67)

Sex	No. of ESBL Isolates	Percentage (%)
Male	36	53.7
Female	31	43.6
Total	**67**	**100**

The sex wise distribution of ESBL positive isolates were shown in Table 3. The isolation rate was more in males 36(53.7%) compared to females 31(46.3%).This is in similarity to the studies which showed males were most commonly affected than females. Males are usually less prone to UTIs as compared to females because of the longer course of urethra and bacteriostatic prostatic secretions. ESBL producing strains can overcome this barrier and hence there is higher incidence in males.

Table 4: Isolation of ESBL Producing Bacteria From Different Wards (n=67)

Name of The Department	No. of ESBL Producing Bacteria	Percentage (%)
Medicine	15	22.4
Urology	11	16.4
Outpatient (O.P.)	10	14.9
Intensive Care Unit (ICU)	9	13.4
Pediatrics	9	13.4
Surgery	8	11.9
Obstetrics & Gynaecology	5	7.5
Total	**67**	**100**

The isolation of ESBL organisms from various wards were shown in Table 4. ESBL strains were isolated predominantly from Medical ward (22.4%) followed by urology ward (16.4%).Majority of ESBL isolates were from Medicine wards. This is in similarity with the study where the isolation was more common from Medicine ward.

Table 5: Comparison of Resistance Pattern of ESBL and Non ESBL *E.coli*

Antibiotics	Total No. of Resistant Microorganisms	ESBL *E.coli* (n=52)	Non-ESBL *E.coli* (n=25)
Ampicillin	62	50 (96.2 %)	12 (48 %)
Gentamycin	15	12 (23.1 %)	3 (12%)
Ciprofloxacin	47	34 (65.9%)	13 (52%)
Amikacin	4	4 (7.7%)	0
Cotrimoxazole	59	44 (84.6%)	15 (60%)
Nitrofurantoin	1	1 (1.9 %)	0
Norfloxacin	60	43 (82.7 %)	17 (68%)
Cefotaxime	54	52 (100 %)	2 (8%)
Cefoperazone Sulbactum	3	3 (5.8%)	0
Amoxicillin clavulanic acid	39	39 (59.6%)	8 (32%)

The resistance pattern of ESBL and non ESBL E. coli were compared in Table 5. The ESBL *Escherichia coli* isolates showed highest resistance towards Cefotaxime 52 (100%), Ampicillin50(96.2%),Cotrimoxazole 44(84.6%), Norfloxacin 43 (82.7%) and Ciprofloxacin 34 (65.9%) compared to non ESBL stains.

Table 6: Comparison of Resistance Pattern of ESBL and Non ESBL *Klebsiella*

Antibiotics	Total Number of Resistant Organisms	ESBL *Klebsiella* (n=10)	Non ESBL *Klebsiella* (n=5)
Ampicillin	14	10 (100%)	4 (80%)
Gentamycin	3	2 (20%)	1 (20%)
Ciprofloxacin	4	4 (40%)	0
Amikacin	2	2 (20%)	0
Cotrimoxazole	11	9 (90%)	2 (40%)
Nitrofurantoin	4	3 (30%)	1 (20%)
Norfloxacin	9	6 (60%)	3 (60%)
Cefotaxime	10	10 (100%)	0
Cefoperazone Sulbactum	-	-	-
Amoxicillin Clavulanic Acid	8	7 (70%)	1 (20%)

The resistance pattern of ESBL and non ESBL *Klebsiella* were compared in Table 6. The ESBL *Klebsiella* strains were highly resistant towards Cefotaxime 10 (100%), Ampicillin 10(100%), Cotrimoxazole 9 (90%), Amoxy clavulanic acid 7(70%) and Norfloxacin 6(60%).

The sensitivity pattern of *Escherichia coli* revealed that maximum sensitivity was shown towards Nitrofurantoin, Cefoperazone sulbactum and Amikacin. Other than beta lactam antibiotics ESBL strains showed maximum resistance towards Gentamycin, Ciprofloxacin, Norfloxacin and Cotrimoxazole. Compared to non ESBL isolates this resistance was statistically significant.

CONCLUSION

Urinary tract infections are the most common infections and urine samples are among the most numerous specimens sent to the laboratory. The aim of the Microbiology laboratory is to reduce morbidity through accurate and timely diagnosis with appropriate antimicrobial sensitivity testing. Compared to double disc approximation test E test was considered the most sensitive method for the confirmation of ESBL organisms. *E.coli* (67.5%)was the most common organism producing ESBLs.The ESBL positivity rate among *Klebsiella* and *Proteus* was 66.7% and 55.6% respectively. Medicine department was the commonest among other departments where ESBL organisms were isolated. ESBL *E.coli* along with cefotaxime showed more resistance to Ampicillin, Cotrimoxazole, Norfloxacin and Ciprofloxacin.ESBL *Klebsiella* showed more resistance towards Ampicillin, Cotrimoxazole and Ciprofloxacin. *Escherichia coli* is the predominant isolate from urine specimens. Multidrug resistance is significantly higher in ESBL positive isolates. Knowledge of the prevalence of ESBLs and resistance pattern of bacterial isolates are important in the prevention.

FUTURE PROSPECTS

Existing of extended spectrum β-lactamases in bacteria and their potential multidrug resistance will create serious problem in the future as their continuous mutation and limited therapeutic option. Indiscriminate use of antibiotics especially 3rd generation cephalosporins and monobactams should beavoided. The regular detection of ESBLs producing organisms should be carried out in every laboratory.

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