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Detection of Extended spectrum beta lactamase, AmpC beta lactamase producing Gram negative bacteria in urinary isolates in a tertiary care hospital

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ABSTRACT:

BACKGROUND: Urinary tract infection (UTI) remains the common infection diagnosed in outpatients as well as in hospitalized patients. Current knowledge on antimicrobial susceptibility pattern of uropathogens is mandatory for appropriate therapy. ESBL, Amp-C and MBL were clinically important cephalosporinases encoded on the chromosomes of many *Enterobacteriaceae* and a few other organisms, which mediates resistance to broad spectrum of antibiotics. Detection of these enzymes can be difficult because their presence does not always produce a resistant phenotype on conventional disc diffusion or automated susceptibility testing methods. These enzymes are often associated with potentially fatal outcome to the individual because of false susceptibility to β-lactams phenotypically in lab reports. Thus, their accurate detection and characterization is important from epidemiological, clinical, laboratory, and infection control point of view. The present study was designed to detect ESBL, Amp-C and MBL from Gram negative bacilli in the urine samples received in Microbiology Department, Govt. Medical College, Thrissur. **AIM:** To detect the ESBL, Amp-C and MBL production from Gram Negative Bacilli isolated in the urine samples. **METHOD:** A total number of 390 non

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repetitive urinary isolates of Gram Negative bacilli were obtained over a period of one year, Isolates resistant to ceftriaxone were tested for ESBL, AmpC, MBL production by double disc synergy test method, Combined disc diffusion method using Cefoxitin (30 mg) dics and confirmed by inhibitor based test using boronic acid as inhibitor and Combined disc diffusion using Imipenem disc and confirmed using EDTA as inhibitor. **RESULTS:** Among these 390 samples 290 (61.7%) were multidrug resistant (MDR) urinary isolates, with predominant of *E.coli*- 298(76.3%) & *Klebsiella spp*- 92 (23.7%). 290 (74.3%) were multi drug resistant, among these 290 resistant strains, 240 (61.5%) isolates were positive for ESBL production, remaining 50 isolates (12.8%) were negative for ESBL production. Out of 240 ESBL producers, 194 were *E.coli* (80.8%) & 46 were *Klebsiella spp* (19.2%). Among the 240 resistant strains, 50 isolates were resistant to Cefoxitin, remaining 190 isolates were sensitive to Cefoxitin. Out of these 50 cefoxitin resistant strain, only 43 were Amp-C producers. Only 7 (1.8%) isolates were MBL producers. **CONCLUSION:** In the present study a large number of uropathogens were found to be ESBL producers. Most of the ESBL producing isolates were multidrug resistant. Monitoring of ESBL, AmpC & MBL productions and antimicrobial susceptibility testing are necessary to avoid treatment failure in patients with UTI. If irrational use of antibiotics is not controlled, infection with these resistant strain will increase, resulting increase morbidity and mortality.

KEY WORDS: ESBL, AmpC, MBL.

Introduction:

Urinary tract infection is among the commonly encountered bacterial infections in human.[1] Urinary tract infections (UTI) are common bacterial infections associated with considerable morbidity and health care cost, with varied clinical spectrum of severity ranging from asymptomatic bacteriuria to cystitis and pyelonephritis to septic shock with multiorgan system failure. UTIs are specially problematic for women, 50-80% of women will suffer at least one episode of UTI in their lifetime and 20 -50% of these women will have recurrent episodes. (2)

ESBLs are strictly defined as β-lactamases capable of hydrolyzing penicillins, broad and extended spectrum cephalosporins and monobactams and are inhibited by clavulanic acid. They

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have been isolated from most of the members of family *Enterobacteriaceae*.[3] ESBLs are derived from genes for TEM and SHV by mutation.(3)

AmpC β-lactamases are clinically important cephalosporins which are encoded on the chromosomes of many *Enterobacteriaceae* and a few other organisms, where they mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and β-lactamase inhibitor/β-lactam combinations. AmpC β-lactamase activity is not affected by the ESBL inhibitor i.e. by clavulanic acid. AmpC enzymes belong to Class-C in the Ambler structural classification of beta lactamases, while they were assigned as Group one in the functional classification scheme of Bush *et al*. Genes for AmpC β-lactamases are may be chromosomal mediated or plasmid mediated in the members of the family *Enterobacteriaceae*. AmpC over production along with porin mutations of the outer membrane, can reduce susceptibility to carbapenems, in particular in plasmid mediated AmpC producers.[4,5,6]

Carbapenems represent as a great advancement for the treatment of serious bacterial infections caused by β-lactam resistant bacteria. Due to their broad spectrum of activity and stability to hydrolysis by most β-lactamases, the carbapenems have been the drugs of choice for treatment of infections caused by ESBL and AmpC Co producing negative bacilli.(7) However, extensive and imprudent use of the carbapenems, poor sanitation and large population has facilitated the emergence of carbapenem resistant bacteria.(8) Resistance to carbapenem is predominantly mediated by metallo-β-lactamases, a class B type of β-lactamases that recognize bivalent metal ions.(7,8) Co-production of multiple beta lactamases in a single isolate limits the treatment options further.[9]

As emerging betalactamases (ESBLs, AmpC and carbapenamases) are main cause of multidrug resistance in gram negative bacilli. The antibiotic option in the treatment of ESBL, AmpC & MBL producing gram negative organisms is extremely limited. Therefore it becomes the necessary to detect these enzymes and to formulate antibiotic policy accordingly. Detection of ESBL/ AmpC/ MBL producing organism from samples such as urine may be important because this represents an epidemiologic marker of colonisation and there is a potential threat for transfer of such organism to other patients.(10) Detection of ESBL, AmpC and MBL is needed to prevent resistant UTIs and to decrease the hospital stay. Considering all these facts, a study is undertaken

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to detect the presence of ESBL, AmpC & MBL in the uropathogens isolated from urine samples received in Microbiology department, Government Medical College, Thrissur.

Materials and Methods

Cross sectional study of 390, non-repetitive clinical isolates of Gram negative bacilli from the urine sample from various departments of Govt. Medical College, Thrissur between January 2016 to December 2016 were included in the study. Sample size was calculated by using the formula $4pq/d^2$.

All Gram negative isolates with significant bacteriuria were included for the study. Culture growth showed Gram positive isolates and Gram negative isolates with insignificant bacteriuria were excluded from the study.

Culture:

Urine samples were inoculated using a standard 4 mm diameter inoculating sterile loop on Mac Conkey agar and Blood agar. The plates were incubated aerobically at 37˚C for 24 hours and were observed for bacterial growth. Culture with significant bacteriuria was further processed. Cultures yielding no growth, insignificant and mixed bacterial growth were excluded. Isolates identified by standard biochemical laboratory methods. Biochemical reactions included Indole test, Urease, Citrate utilization, Triple sugar iron agar, Mannitol motility medium, Nitrate reduction test, 1% Sugars- Glucose, Lactose, Sucrose and Mannitol.(11)

Antimicrobial susceptibility tests:

Antibiotic susceptibility testing was done according to CLSI recommended Kirby-Bauer disc diffusion method. Antibiotic discs from Hi media(Mumbai) were used. The antibiotic discs used were Ampicillin (25 µg), Gentamicin (10µg), Cefazolin (30µg), Ciprofloxacin (5µg), Amikacin (10µg), Ceftriaxone (30µg), Co-trimoxazole (25µg), Nitrofurantoin (30µg), Norfloxacin (30µg), Imipenem (10µg), Cefepime (30µg), Piperacillin-tazobactam (100/10µg). The results were interpreted as per CLSI (Clinical laboratory Standard Institute, CLSI 2016) guidelines.(12-CLSI).

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ESBL Screening:

The isolates that show resistance to 3rd generation cephalosporins- Ceftazidime or Ceftriaxone by standard disc diffusion method were selected for the study of ESBL and Amp- C production. As proposed by CLSI, M100-S16 document, the use of more than one indicator cephalosporins suggested was improve the sensitivity of detection. In the present study Ceftazidime (30µg), Ceftriaxone (30µg), Cefotaxime(30µg) and Aztreonam (30µg) was used for screening.(12)

DETECTION OF ESBL

DOUBLE DISC APPROXIMATION TEST:

A 0.5 McFarland standard inoculum of test organism was prepared and swabbed on a Mueller-Hinton agar plate. Antibiotic discs of Cefotaxime (30µg), Ceftazidime (30µg), Cefepime (30µg), and Aztreonam (30µg) were placed 20mm apart from Amoxicillin/Clavulanate (20/10µg) disc and incubated for 24 hours. Organisms that showed an enhancement of the zone of inhibition towards the disc containing clavulanate were considered as ESBL producers. [12]

Phenotypic confirmatory test for ESBL detection:

Isolates that were considered to be positive for ESBL production by the screening test were subjected to the Phenotypic Confirmatory Test (CLSI-PCT) as recommended by 2016 CLSI guidelines. From the pure cultures of bacteria grown overnight on MacConkey agar, a suspension matching 0.5 McFarland standard (1.5 x 10^8 CFU/ml) was made in peptone water. Using sterile cotton swab, the bacteria were spread on Mueller Hinton agar to obtain a lawn culture. After allowing the plate to dry, discs of ceftazidime (30 µg) (CAZ), Ceftazidime + Clavulanic acid (30/10 µg) (CAC), Cefotaxime (30µg) (CTX), Cefotaxime + clavulanic acid (30/10µg) (CEC) were placed on the surface and the plates were incubated in air at 37˚C for 18-24 hours. Following growth, the diameter of the zones around the disks were measured and recorded. An increase in the zone diameter by ≥5 mm around the discs containing Cephalosporin with Clavulanic over the disks containing cephalosporin alone indicated ESBL production.(12)

Phenotypic confirmatory test for AmpC detection:

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Isolates that were positive in the initial screening test but negative in the subsequent ESBL phenotypic confirmatory test were tested for AmpC beta-lactamases. [13] Inhibition of AmpC beta-lactamase was achieved by adding 400 µg of amino-phenylboronic acid to antibiotic discs. The stock solution of amino-phenylboronic acid was prepared by dissolving 120 mg of amino-phenylboronic acid in 3 ml of dimethyl sulfoxide. Three milliliters of sterile distilled water was added to this solution. Twenty microliters of the stock solution was dispensed onto discs containing cefoxitin (30µg). From the pure cultures of bacteria grown overnight on MacConkey agar, a suspension matching 0.5 McFarland standard (1.5 x 10^8 CFU/ml) was made in peptone water. Using sterile cotton swab, the bacteria were spread on Mueller Hinton agar to obtain a lawn culture. Cefoxitin, Cefoxitin + amino-phenylboronic acid discs were placed at a distance of 20 mm and incubated aerogenically at 37˚C for 18-24 hours. An increase in inhibition zone diameter by ≥5 mm around the disc containing Cefoxitin and amino phenylboronic acid indicates Amp-C producers.(13)

DETECTION OF ESBL & AmpC Co-PRODUCERS:

Isolates that were positive in the initial screening test but negative in the subsequent ESBL phenotypic confirmatory test were tested for co-production of AmpC beta-lactamases as described by Coudron [13]. Inhibition of AmpC beta-lactamase was achieved by adding 400 µg of amino-phenylboronic acid to antibiotic disks. The stock solution of amino-phenyl boronic acid prepared by dissolving 120 mg of amino-phenyl boronic acid in 3 ml of dimethyl sulfoxide. Three milliliters of sterile distilled water added to this solution. Twenty microliters of the stock solution dispensed onto disks containing cefotaxime + clavulanic acid (30/10µg) and cefotaxime (30µg). Disks were allowed to dry for 30 minutes and stored in airtight vials with desiccant at 4˚C. From the pure cultures of bacteria grown overnight on MacConkey agar, a suspension matching 0.5 McFarland standard (1.5 x 10^8 CFU/ml) was made in peptone water. Using sterile cotton swab, the bacteria were spread on Mueller Hinton agar to obtain a lawn culture. Cefotaxime, Cefotaxime + Clavulanic acid, Cefotaxime + Clavulanic acid + Amino-phenylboronic acid, Cefotaxime + Amino-phenylboronic acid disks were placed on the surface of the MHA plates and incubated at 37˚C for 18-24 hours.(13)

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Table 1: Interpretation Of ESBL and AmpC Co-producers:

CTX	CTX+CLA	CTX+CLA+PBA	CTX+PBA	INTERPRETATION
R	+	NA	NA	ESBL only
R	-	+	+	Amp-C only
R	-	+	-	ESBL & Amp-C Co-Producers

CTX - cefotaxime, CLA - clavulanic acid, PBA – aminophenyl boronic acid, R = resistant

NA= Not applicable,

- *(+) → Indicates an increase in inhibition zone diameter by ≥5 mm*
- *(-) → Indicates increase in inhibition zone diameter by <5 mm (13)*

PHENOTYPIC COMBINED DISC TEST FOR DETECTION OF MBL:

To make 0.5 M Ethylene diamine tetra acetic acid (EDTA) solution was prepared by dissolving 18.6 g of disodium EDTA.$2H_2O$ in 100 mL of distilled water, and the pH was adjusted to 8 by sodium hydroxide (NaOH). Then the mixture was sterilized in the autoclave to prepare a sterile EDTA solution. To prepare EDTA-containing Imipenem disks (750 g EDTA); 10 µL of EDTA solution was added to Imipenem disks (10g). These disks were dried immediately in a 37°C incubator and stored at 4°C in airtight vials until use. For each isolate, 1 imipenem disc and 1 EDTA containing Imipenem disc were placed at a suitable distance on the surface of Mueller-Hinton agar plates inoculated with a bacterial suspension equivalent to 0.5 McFarland Standard. After 24 hours of incubation at 37°C, an increase of ≥7 mm in the inhibition zone diameter of EDTA containing Imipenem disk compared to Imipenem disk alone was considered to be a positive test for the MBLs producer Imipenem resistant strains. [14]

Results:

This cross sectional study was carried out in the Department of Microbiology, Govt. Medical College, Thrissur, for the period of one year from January – December 2016. Among the 390 GNB isolates from urine, Majority of the isolates were *Esch.coli* 298(76.3%) followed by *Klebsiella* spp 92(23.7%). Among 390 Gram negative samples, majority of the growth had

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attained in female (61.7%) than male, which contributed 38.3%. Females are commonly affected than males.

In age wise distribution of UTI, most common age group affected was above 60 years which contributed 131(33.7%) followed by 46-60 years age group which about 25.2%.

Out of 390 samples, 290(74.3%) isolates were multi drug resistant, of which 240 (61.5%) isolates were positive for ESBL production, remaining 50 isolates (12.8%) were negative for ESBL production. Out of 240 ESBL producers, 194 were *Esch.coli*(80.8%) & 46 were *Klebsiella* spp (19.2%).

Among the 240 resistant strains, around 190 isolates were sensitive to cefoxitin and 50 isolates were resistant to Cefoxitin which was showed screening positive for AmpC production. In which 43 isolates showed confirmed AmpC production by Phenotypic confirmatory test. Out of which 29 isolates were *Esch.coli* and 14 isolates were *Klebsiella* spp.

There were 8(2%) isolates in the present study were ESBL and Amp-C Co-producers. There were 7(1.8%) MBL producers were identified. In which, 6 isolates were *Klebsiella* spp & 1 isolate was *E.coli.* The results were statistically significant for the resistance of the above mentioned antibiotics. Chi-square test and $p<0.01$ applied.

Discussion:

The morbidity and mortality rate in the hospital setting has been alarmingly increasing since the infections associated with multidrug resistant bacteria that produce various beta-lactamases enzymes have been increasingly reported. The most common strains which beta lactamase enzymes producers were found in members of *Enterobactericeae* family.

Majority of the isolates were *Esch.coli* and *Klebsiella* spp., which account for 76.3% and 23.7% respectively. Similar report was found by Pushpa *D* et al, where they reported *Esch.coli* and *Klebsiella* spp were most common isolates which account for 63.8% and 36.2%. Thus our study correlates with the study conducted by Pushpa D et al. [15]

The most common age group affected by UTI was above 60 years (33.7%) followed by 46-60 years which was 25.2%. In a study by *Eshwarappa* et al,[16] a similar finding was recorded with

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most of UTI seen in the elderly age group above the age of 60. This may be due to hormonal changes and stress.

From the total of 390 samples, ESBL producing strains were higher among females than males. Rodr'iguerz –Banto J et al in their study on ESBL, have mentioned female sex and elderly age group are risk factors for acquiring community associated ESBL infections.[17] UTI is predominantly a disease of females. The incidence of UTI among males remains relatively low after 1 yrs of age.[18]

Most of the isolates were resistant to Ampicillin, Cephazolin, Ceftriaxone, Ceftazidime, and Aztreonam. Resistant percentage of Ceftriaxone, Ceftazidime, Aztreonam were 72.2%, 71.9%, 71.9% respectively. It correlates with the study done by Sasirekha et al & Singh NP et al, In their study they reported a resistance of 84% ,75%, 85% resistance to Cefotaxime, Ceftriaxone and Ceftazidime respectively.[19,20] The emergence of antibiotic-resistant organisms in hospitals is concert with the use of high levels of antibiotics use caused the emergence of resistant organisms and they might be inherently more virulent than the organisms which were sensitive.

In present study 240 (61.5%) isolates were positive for ESBL production, remaining 50 isolates (12.8%) were negative for ESBL production. Out of 240 ESBL producers, 194 were *E.coli* (80.8%) & 46 were *Klebsiella spp* (19.2%). A study from Nagpur (Supriya et al), *E. coli* was found to be the most common organism followed by *Klebsiella pneumoniae* (82%, 37.8%) [21]. A study by Bhattacharjee et al. in which ESBL was observed in 62.7% *Klebsiella pneumonia* and 46.5% of *Escherichia coli*.[22] Looking at overall trend of ESBL is on the rise; This is due to irrational and wide use of third generation Cephalosporins in both the hospital and community and is believed to be the major cause of mutations in these enzymes that has lead to the emergence of the ESBLs.[23]

In this study most of the ESBL producing organisms were found to be co-resistant to Fluroquinolones, Aminoglycosides and Co-trimoxazole, which correlates with the study done by Denholm et al and Jabeen et al.[24,25] This was due to the genes encoding these beta lactamases were often located on large plasmids that also encode genes for resistance to others antibiotics, including Aminoglycosides, Tetracycline, Sulfonamides, Trimethoprim and Chloramphenicol.[25]

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There were 50 isolates resistant to Cefoxitin detected by AmpC screening test. In which, 43(11%) isolates were confirmed by phenotypic confirmation test. Among Amp-C positive isolates, 29(7.5%) were *E.coli* and 14 (3.5%) were *Klebsiella spp.*

In another study by Rathna et al from Karnataka, 3.3 percent of the isolates produced Amp-C beta lactamases.[26] Thus our study was correlating with study conduced by Rathna et al. In contrast to the above, a higher percentage of Amp-C detection is mentioned in studies by Patel et al. from Ahmadabad which account of 45%.[27] Multidrug resistance was high among the Amp-C producing isolates suggesting plasmid-mediated spread. Plasmid-mediated Amp-C genes are of special interest because their mobility allows them to emerge in one genus or species and spread to different organisms. [28]

There were 8(2%) isolates in the present study were ESBL and Amp-C Co-producers. In a study conducted by Ananthan S[29] et al., showed that there were 37.2 isolates(*Esch.coli* & *Klebsiella* spp) were ESBL & AmpC Co-producers. Higher rate of AmpC production was found due to loss of outer membrane protein. They further made a note that Cefoxitin resistant Amp-C positive isolates showed loss of porin protein, indicating that Cefoxitin resistance was mediated by both Amp-C beta lactamase production and loss of Outer membrane protein. [29]

Only 7 (1.8%) isolates were MBL producers, among this 7 isolates, 6 isolates were *Klebsiella Spp* and 1 isolate was *E.coli.* Enwuru et al. from Nigeria reported that among Gram- negative bacterial strains tested (23%) were confirmed to be MBL producer, in this *E. coli* (50%) and *Klebsiella spp*. (36%).[30] Carbapenems have a broad spectrum of antibacterial activity, often used as a last resort in infections caused by multi drug resistant Gram negative bacilli.[31] MBLs producing Gram-negative bacteria are an increasing public health problem worldwide because of their resistance to all β lactam antibiotics.[30] MBL genes were typically carried on transferable plasmids or were part of the bacterial chromosome, was primarily detected in *Pseudomonas aeruginosa* but was later found in other Gram negative bacteria, non-fermenters and members of the *Enterobacteriaceae* family.[31]

Conclusion:

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Early identification of *E.coli* and *Klebsiella* strains that produce beta lactamase is crucial for lowering morbidity and death rates as well as preventing the spread of these bacteria throughout the population. The current study found that *E.coli* and *Klebsiella* were manufacturing more ESBL, AmpC, and MBL. This study emphasizes the serious threat posed by the impending evolution of microorganisms resistant to most drugs. The fast global spread of *E.coli* and *Klebsiella* that produce ESBL/AmpC/MBL, especially in India, suggests that robust infection control strategies and ongoing monitoring systems are important.

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