3.1 INTRODUCTION

Beta – lactam antibiotics are a broad class of antibiotics which include pencillin derivatives, cephalosporins, monobactam, carbapenems and beta-lactamase inhibitors; basically any antibiotics agent which contains a β lactam nucleus in its molecular structure. They are the most widely used group of antibiotics available. While, traditionally β lactam antibiotics are mainly active only against gram – positive bacteria, the development of board – spectrum β lactam antibiotics against various gram – negative organisms has increased the usefulness of the beta – lactam antibiotics.

Group of β lactams

The β lactams are a family of antimicrobial agents consisting of four major groups: penicillins, cephalosporins, monobactams, and carbapenems. They all have a β lactam ring, which can be hydrolyzed by β lactamses.

3.1.a Mode of action

β lactam antibiotics are bacterial, and act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity, especially in gram – positive organisms. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as pencillin binding proteins (PBP).

β lactams antibiotics are analogues of D – alanyl – D- alanine – the terminal amino acid residues on the precursor NAM/NAG – Peptide subunits of the nascent peptidoglycan layer. The structural similarity between β lactam antibiotics and D –
alanyl –D – alanine facilitates their binding to the active site of penicillin binding proteins (PBPs).

The β-lactam nucleus of the molecule irreversibly binds to (acylates) the Ser403 residue of the PBP active site. This irreversible of the PBPs prevents the final crosslinking (transpeptidation) of the nascent peptidoglycan layer, disrupting cell wall synthesis. Inhibition of PBPs may also lead to the activation of autolytic enzymes in the bacterial cell wall.

3.1.b Mode of resistance in Beta-lactam antibiotics

By definition, all Beta-lactam antibiotics have a β-lactam ring in their structure. The effectiveness of these antibiotics relies on their ability to reach the Penicillin Binding Proteins (PBPs) intact and their ability to bind to the PBP. Hence, there are 2 main modes of bacterial resistance to beta-lactams.

The first mode of Beta-lactam resistance is due to enzymatic hydrolysis of the β-lactam ring. If the bacteria produce the enzymes beta-lactamase or penicillinase, these enzymes will break open the β-lactam ring of the antibiotic, rendering the antibiotic ineffective. The genes encoding these enzymes may be inherently present on the bacterial chromosome or may be acquired via plasmid transfer, and beta-lactamase gene expression may be induced by exposure to beta-lactams.

However, in all cases where infection with beta-lactamase-producing bacteria should be suspected, the choice of a suitable beta-lactam antibiotic should be carefully considered prior to treatment. In particular, choosing appropriate Beta-lactam antibiotic therapy is highly important against organisms within ducible beta-lactamase expression.

Second mode of beta-lactam resistance is due to possession of altered penicillin binding proteins. The β-lactams cannot bind as effectively to these altered PBPs, and as a
result, the beta-lactams are less effective at disrupting cell wall synthesis. Notable examples of this mode of resistance include methicillin-resistant *Staphylococcus aureus* (MRSA) and penicillin-resistant *Streptococcus pneumoniae*. Altered PBPs do not necessarily rule out all treatment options with Beta-lactam antibiotics.

### 3.1.c Beta lactamases

Resistant bacteria are emerging worldwide as a threat to favorable outcome of common infections in community and hospital settings. Beta-lactamase production by several gram negative and gram positive organisms is perhaps the most single mechanism of resistance to penicillin cephalosporin.

#### Synthesis and mode of transfer

The synthesis of β-lactamases is either chromosomal (constitutive), as in *Psuedomonas aeruginosa*, or plasmid mediated (inducible), as in *Aeromonas hydrophila* and *Staphylococcus aureus*.

#### Location

In the Gram positive bacteria β-lactamases are secreted to the outside membrane environment as exoenzymes. In the Gram negative bacteria they remain in the periplasmic space, where they attack the antibiotic before it can reach its receptor site. (Bradford *et al.*, 1994).

### 3.1.d Mechanisms of action

They contain an active site consisting of a narrow longitudinal groove, with a cavity on its floor (the oxygen pocket), which is loosely constructed in order to have conformational flexibility in terms of substrate binding. Gaillot *et al.*, (1998).
3.2 Extended spectrum beta-lactamase (ESBL)

The persistent exposure of bacterial strains to a multitude of β-lactams has induced a dynamic and continuous production and mutation of β-lactamases in these bacteria, expanding their activity even against the third and fourth generation cephalosporins such as ceftazidime, cefotaxime, and cefepime and against aztreonam. Thus these new beta-lactamases are called extended spectrum beta-lactamases. The incidence of ESBL producing strains among clinical isolates has been steadily increasing over the past few years resulting in limitations of therapeutic options (Ananthakrishnan et al., 2001).

These ESBLs enzymes are plasmid borne and have evolved from point mutations altering the configuration of the active site of the original and long known beta-lactamases designated TEM-1, TEM-2, and SHV-1. The activity of these enzymes is limited to ampicillin, penicillin, and carbenicillin. ESBL mediated resistance was 25.8% in Chennai (Subha et al., 2002).

The original TEM was first discovered in E. coli is named after a patient named Temoniera in Greece from Whom the pathogen was isolated but it spread rapidly to other bacteria. Although TEM-type beta-lactamases are most often found in E. coli and K. pneumoniae they are also found in other genera of Enterobacteriaceae and in other penicillin or ampicillin resistant Gram negative bacteria such as Haemophilus influenzae and Neisseria gonorrhoeae (Medeiros et al., 1984).

The SHV enzymes, named after the “sulphydryl variable” active site, are commonly associated with K. pneumoniae. At first these bacteria contained a Single ESBL gene, but now multiple ESBL genes are commonly present in a single strain, further complicating the process of detecting them and identifying an appropriate
treatment regimen (Bradford et al., 1994). To date, more than 90 TEM-type and more than 25 SHV-type beta-lactamases have been identified (Bradford et al., 1994-2001).

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, amino glycosides, and trimethoprim-sulfamethoxazole.

β-lactamase inhibitors such as β-lactam-β-lactamase inhibitor combinations could show higher in vitro susceptibility results against bacterial strains with ESBL production than their original parent. However, their in vivo activity remains to be validated (Ferrara et al., 2000).

Infections with ESBL producing bacteria can result in avoidable failure of treatment and increased cost in patients who have received inappropriate antibiotic treatment. Nosocomial outbreaks of this form of resistance are most often associated with intensive care units and oncology, burns, and neonatal wards. They can result in prolongation of hospital stay as well as devastating or even fatal consequences (Moland et al., 1998).

3.2. a Structure of Beta-lactamase and Mechanism of action (ESBL)

All ESBLs have serine at their active site except for small (but rapidly growing) group of metallo β lactamases belonging to class B. They share several highly conserved amino acid sequences with penicillin binding proteins.

β-lactamase attack the amide bond in the Beta-lactam ring of penicillins and cephalosporins with subsequent production of penicillanoic acid and cephalosporic acid respectively, ultimately rendering the compounds antibiotically inactive (Ambler et al., 1980).
The most frequent co-resistance found in ESBL producing organisms are aminoglycosides, fluoroquinolones, tetracycliness, chloromphenical and sulfamethaxazole-trimethoprim.

### 3.2.b Methods of detecting ESBLs

The increasing prevalence of ESBL producing bacterial strains caused many outbreaks. This has warranted the establishment of rapid and reliable laboratory methods for screening and confirmation.

Generally, an isolate is suspected to be an ESBL produce when it shows in vitro susceptibility to the second generation cephalosporins (cefoxitin, cefotetan) but resistance to the third generation cephalosporins and to aztreonam. Moreover, one should suspect these strains when treatment with these agents for Gram negative infections fails despite reported in vitro susceptibility. Once an ESBL producing strain is detected, the laboratory should report it as “resistant” to all penicillins, cephalosporins, and aztreonam, even if they test as susceptible. Other antimicrobial agent can be reported as they are tested.

### 3.2.c Treatment of ESBLs

Essentially, the choice of drug for treating ESBL producing bacteria is limited to carbapenems-for example, imipenem. Alternatively, fluoroquinolones and amino glycosides may be used if they show in vitro activity. Although clinical data for their use are absent, a beta-lactam-beta-lactamase inhibitor combination such as amoxicillin-clavulanate or piperacillin-tazobactam may also be a fluther option to consider. All these agents should be used with caution, however, as their susceptibility varies among ESBL producers. Cefamycins, such as cefoxitin and cefotetan, although active in vitro, are not recommended for treating such infections, because of the relative case with which these
strains decrease the expression of outer membrane proteins, rendering them resistant (Yuan et al., 1998).

3.3 MATERIAL AND METHODS

3.3.a Collection of sample

Urine was collected in sterile, dry, wide necked, leak proof container. About 20ml of urine sample was collected. The patients were instructed to collect mid stream urine. First voided urine was not collected because it may be contaminated with microbes from lower portion of urethra.

The samples were delivered immediately. The urine was refrigerated at 4°C. If the delay was more than 1 hour, boric acid should be added. Specimen containing boric acid need not be refrigerated.

3.4 PROCESSING THE SAMPLE

3.4.a Macroscopic observation

The appearance (i.e.) color and nature of the sample recorded.

Normally freshly passed urine was clear and pale yellow-to-yellow. Cloudiness may develop due to precipitation of urates in acid urine or phosphates and carbonates in alkaline urine. Urates may give the urine a Pink-Orange color.

3.4.b Microscopic Observation

Direct Smear Examination

A drop of the centrifuged urine sample was stained (Gram’s staining). The prepared slide was examined under oil immersion to identify the bacteria, White cells, pus cells, red cells, yeast cells, epithelial cells, cysts, crystals and parasites.
Culture of the sample

For selective isolation, mix the urine was mixed well. A loopful of the well urine sample from the well was streaked on Nutrient agar, Macconkey agar and blood agar. Inoculated plates were incubated at 37°C for 24-48hrs and examined for bacterial growth. Nutrient agar media was prepared to infer the growth of bacteria. Macconkey agar medium was used to differentiate the growth of Gram negative bacteria. Blood agar was used to infer the presence of Gram positive and hemolytic bacteria.

3.5 IDENTIFICATION OF MICROORGANISMS

The colonies formed on various agar plates were examined.

3.5.a Gram's staining

A loopfull of overnight broth culture was smeared on a glass slide and beat fixed. The smear was flooded with crystal violet. After 1 minute, crystal violet was washed with tap Water and floods the smear with Gram's iodine (mordant). After 1 minute, the grame's iodine was washed away with distilled distilled water and decolorizer ethyl alcohol (95%) added. The counter stain safranin was added after washing the decolorizer. After 45 seconds, the smear was washed. The slides were dried and examined under oil immersion.

3.5.b Motility (Hanging drop technique)

A coverslip Was taken and its edges were greased by vasline. using sterile technique, a loopfull of the culture was placed in the center of clean coverslip. Cavity slide was inverted over the coverslip. This set up was observed for motility.
3.5.c Biochemical analysis

**IMVIC Test**

**Indole production test.**

The isolated test cultures were inoculated into tryptophan enriched peptone Water and incubated at 37°C for 24-48 hours. To this, 0.5ml of Kovac’s reagent was added. Positive test (indole produced) was indicated by the appearance of red ring in alcohol layer.

**Methyl red test**

The glucose phosphate peptone water was inoculated with the isolated test organisms and incubated at 37°C for 24 hours. A few drops of methyl red indicator was added to the incubated culture.

A positive test (production of sufficient acid turning the medium acidic pH 4.5 or less) is indicated by the retention of the red color of the indicator turning the medium into a bright red color. A change to yellow colour indicates that the pH of the medium is greater than 6.0, a negative methyl red test.

**Voges-Proskauer test**

The isolated test culture was inoculated into glucose phosphate peptone water and incubated at 37°C for 48 hours. 1ml of 40 NaOH and 3ml of 5 alpha-naphthol solution in absolute ethanol were added to the incubated culture.

A positive reaction is indicated by the development of a pink color with in few minutes.
3.5.d Citrate utilisation test

The pH of the medium was adjusted to 6.9. It was autoclaved and into tubes and allowed to set as slants. The test culture was inoculated into the prepared slants and incubated at 37°C for 24-96 hours.

A positive test is indicated by a steam of growth and change in color from green to blue.

3.5.e Triple Sugar- Iron test

Triple Sugar-Iron agar was sterilized and dispensed into tubes and set as slants. The test organism was stabbed into the deep of the agar and streaked on the slant surface. The tubes were incubated at 37°C for 24 hours.

Results were interpreted as follows

a. Alaline slant / Acid butt (K/A)
   Glucose but not lactose or sucrose fermented.

b. Alkaline slant/ Acld (black) butt (K/A/HS)
   Glucose but not lactose (or sucrose) fermented with Hydrogen sulphide production.

c. Acid slant / Acid butt (A/A)
   Glucose and lactose or sucrose fermented

d. Alkaline slant / Alkaline butt (K/K)
   Glucose, lactose and sucrose not fermented.

3.5. f Urease test

Christensen's urea agar base was prepared without glucose and urea and sterilized by autoclaving at 121°C for 30 minutes. Glucose and urea solutions were sterilized separately and added to molten cooled base medium at 50°C. It was then dispensed into
sterile tubes and allowed to set as slants. The isolate was streaked into the prepared slants and incubated at 37°C for 24-96 hours.

Urease positive cultures change the color of the indicator to purple pink.

3.5.g Oxidase test

Reagent: 1 % tetra methyl paraphenylene diamino dihydro chloride.

Uniform discs were punched out from Whatmann filter paper No. 1 and sterilized at 160°C for 1 hour in hot air oven. Discs were then soaked in freshly prepared 1% oxidase reagent and allowed to dry. Discs which have a light purple tint were stored in a dark bottle at room temperature.

At the time of use, a colony of the test organisms was smeared on a disc placed on a clean glass slide and observed.

A positive oxidase test was indicated by the development of a deep purple blue within 5-10 seconds.

3.5.h Catalase test

Reagent: 3.0% hydrogen peroxide

A small amount of pure growth was transferred into the surface of a clean, dry glass slide and a drop of 3 % hydrogen peroxide was placed over it.

A positive catalase test was indicated by the evolution of bubbles.

Isolated colonies are identified by performing tests like gram's staining, motility, urease, oxidase, triple sugar iron test IMVic tests whenever necessary.

3.6 ANTIBIOGRAM

Peptone water was inoculated with 4-5 colonies of the isolates and incubated 4-6hrs at 37°C. Mueller-Hinton agar medium was used for antibiogram. Antibiogram was done by Kirby-Bauer disc diffusion technique. The media was inoculated by carpet
method. Antibiotic discs were placed over the media and inoculated for overnight at
37°C. Antibiotic discs used for this antibiogram study were Gentamycin, Ofloxacin,
Ampicillin, Ciprofloxacin, Sparfloxacin, Sulcef, Amikacin, Gatifloxacin and
Tobramycin (Hi-Media, Bombay).

3.6.a Detection of Extended Spectrum Beta-Lactamase (ESBL) production

Several methods had been developed to confirm the presence of ESBL activity on
the isolates.

3.6.b Microbiological Methods:

- Clover glass method
- Disc comparator method.
- Double Disc Diffusion Synergy Test (DDST)
- Modified Double Disc approximation Method (MDDM).
- Modified three dimensional Method.
- Inhibitor potentiated disc diffusion method.
- MIC reduction test.
- Vitek ESBL test.
- E-test.
- Novel scheme of disc placement method (Rodrigues et al).

3.6.c Biochemical Methods:

- 1. Acidometrio methods.
- Mierotitre method.
- Membrane method
- 2. Iodométrio methods.
In this study, double disc diffusion synergy Test (DDST) method was followed.

1. In this test, the organism was swabbed onto a Mueller-Hinton agar plate.

2. A susceptibility disk containing amoxicillin-clavulanate (Augmentin) was placed in the center of the plate.

3. The antibiotic disks containing oxyimino-B-lactam such as Cefotaxime, Ceftriaxone, Cefotaxime and Cefpodoxime (Hi-Media Bombay) were placed 15 mm (center to center) from the amoxicillin-clavulanate disk.

4. Enhancement of the zone of inhibition of the oxyimino-beta-lactam in Cefotaxime, Ceftriaxone, Cefotaxime and Cefpodoxime caused by the synergy of the clavulanate in the amoxicillin-clavulanate disk was a positive result.

3.7 RESULT AND DISCUSSION

The present study was conducted to find out the prevalence of extended Spectrum of Beta Lactamase (ESBL) activity among the gram negative uropathogens.

The study group involved 132 patients, which comprised 74 males and 58 females. The age groups were recorded and tabulated.

Table: 3.1ESBL Production among gram negative uropathogens

<table>
<thead>
<tr>
<th>Species of Gram negative bacteria</th>
<th>Total number n=55</th>
<th>Suspected ESBL Producer n=43</th>
<th>ESBL producer n=23</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>18</td>
<td>16</td>
<td>10</td>
<td>55.56</td>
</tr>
<tr>
<td><em>Klebsiella sp</em></td>
<td>12</td>
<td>10</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td><em>Enterobacter sp</em></td>
<td>10</td>
<td>8</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td><em>Citrobacter sp</em></td>
<td>9</td>
<td>6</td>
<td>2</td>
<td>22.22</td>
</tr>
<tr>
<td><em>Proteus sp</em></td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>33.33</td>
</tr>
</tbody>
</table>
Antibiogram:

Antibiotic sensitivity test was done for all 55 gram negative uropathogens. Non beta-lactam antibiotics were used for the antibiogram. Amikacin and Sulcef was highly sensitive (76.36%) among all gram negative uropathogens. The sensitive nature of other antibiotics against gram negative bacteria were also recorded and tabulated. Sensitivity of non beta-lactam antibiotics against ESBL positive were also recorded in the table.

Control measures

Proper infection control practices and barriers are essential to prevent the spreading and outbreaks of ESBL producing bacteria. The reservoir for these bacteria seems to be the gastrointestinal tract of patients. Alternative reservoirs could be the oropharynx, colonized wounds, and urine. The contaminated hands and Stethoscopes of healthcare providers are important factors in spreading infection between patients. Essential infection control practices should include hand washing by hospital personnel, increased barrier precautions, and isolation of patients colonized or infected with ESBL producers. Other practices that have minimized the spread of such organisms include clinical and bacteriological surveillance of patients admitted to intensive care units and antibiotic cycling, as well as policies of restriction, especially on the empirical use of broad spectrum antimicrobial agents such as the third and fourth generation cephalosporins and imipenem (Pena et al., 1998).

Urinary Tract Infections (UTIs) are frequent causes of morbidity in out patients as well as most frequent manifestation of nosocomial infections. The incidence of ESBL producing strains among clinical isolates has been steadily increasing over the past years resulting in limitation of therapeutic options (Sumeeta et al. 2002).

Micro organisms responsible for urinary tract infection (UTI) such as *Escherichia coli* and *Klebsiella sp* have the ability to produce ESBLs in large quantities. These
enzymes are plasmid borne and confer multiple drug resistance, making urinary tract infection difficult to treat.

Information on the prevalence of ESBL producers in people suffering with urinary tract infection is limited, this study was undertaken to determine the occurrence of ESBL producers causing UTI in a tertiary care hospital in Tirunelveli District of Tamilnadu and to study the anti microbial susceptibility pattern of ESBL producers and non producers.