**Phenotypic characterization of aerobic gram-negative bacilli with the importance to ESBL and AmpC beta-lactamases**

**Abstract**

The most frequent form of beta-lactam resistance is represented by beta -lactamases. Along with inducible AmpC  beta-lactamases and derepressed mutants, extended spectrum beta-lactamases (ESBLs) are a significant group of beta-lactamses that are currently being discovered in great numbers throughout the world. The current work was conducted to precisely examine ESBLs, AmpC beta-lactamases (both inducible and hyperproducers i.e., derepressed mutants), and beta lactamase production in clinical isolates by rearranging conventional discs used in reporting susceptibility. The two most common bacteria found in mid stream urine (MSU) samples around the world are Escherichia coli and Klebsiella pneumoniae. These uropathogens are mostly blamed for being the main manufacturers of extended spectrum beta-lactamase (ESBL), which severely restricts the therapeutic therapy of urinary tract infections. Antibiotic resistance among bacterial strains is a growing global issue. In both the community and hospital settings, urinary tract infections (UTIs) are among the most prevalent bacterial illnesses in people. The two most prevalent pathogens identified from urine are Escherichia coli and Klebsiella pneumoniae. Beta-lactamases are still the main factor in gram-negative bacteria developing resistance to beta-lactam antibiotics. In this research paper Antibiotic sensitivity testing by Kirby- Bauer method and screening tests and confirmatory test were done. Screening test and confirmatory test for AmpC beta was also done.

**Key words:** Beta –lactamases, Escherichia coli, Klebsiella pneumoniae, Antibiotic resistance, Kirby- Bauer method.

­­­**Introduction**

Aerobic Gram-negative bacilli are the majority of bacterial isolates found in clinical collections, with gram-positive bacteria making up a smaller portion [1], [2]. They can be found in any kind of infectious disease, and have been linked to antibiotic resistance. They are found in both human and animal big intestines and can be found on the outside, within, and in the environment of man [3], [4],[5],[6].Non-fermentative Gram-negative bacilli do not use carbs as a source of energy or break them down by metabolic processes other than fermentation [7],[8],[9]. Beta-lactamases hydrolyze beta-lactam antibiotics, rendering them inactive and giving rise to inactive substances. Some bacteria develop beta-lactamases, making them resistant to beta-lactam antibiotics [10],[11],[12].Beta lactamases are the main contributor to Gram negative bacteria developing resistance to beta lactam antibiotics. Cephalosporins were first used in clinical settings in the early 1980s to combat beta-lactamase-mediated bacterial resistance to antibiotics[13],[14],[15]. This was hailed as a victory in the struggle against beta-lactamase-mediated bacterial resistance to antibiotics [16], [17], [18].Beta lactamases, such as ESBLS, have evolved due to the widespread use of newer-generation cephalosporins [19],[20],[21]. Transferrable conjugative plasmids used to make ESBLs often contain resistance genes for other antimicrobial drugs, leading to the spread of resistance to other Gram negative bacilli in hospitals and the general population [22], [23], [24],[25]. Because they are frequently missed by routine susceptibility testing techniques, ESBL-producing strains are probably more common than is currently recognized. ESBL-producing bacteria with an incredibly broad spectrum of antibiotic resistance have been recently identified in reports [26],[27],[28],[29]. The bacterial enzymes known as beta lactamases deactivate beta lactam antibiotics through hydrolysis, producing inactive molecules [30],[31],[32]. Some bacteria develop beta-lactamases, which make them resistant to beta lactam antibiotics such as Penicillin, Cephalosporin, Cephamycin, and Carbapenems [33]. These antibiotics all share the same component in common with one [34],[35]. From both a therapeutic and infection control perspective, immediate action must be taken due to the high incidence of beta-lactamase synthesis in clinic isolates caused by numerous mechanisms.When taken orally, beta lactamases may have clinical advantages in maintaining the normal intestinal flora during parenteral antibiotic therapy [36],[37],[38]. A wide variety of nosocomial pathogens may be protected against by this. The rise of broadened spectrum cephalosporin resistance in Gram  negative bacteria has been a major source of worry [39],[40]. The penicillin, cephalosporin, carbapenem, and monobactam families of antibiotics, which are also known as beta-lactam antibiotics, are the main groups that include the beta-lactam ring [41],[42],[43]. These antibiotics function by preventing bacteria from synthesising cell walls. Bacteria, especially Gram-positive ones, are fatally affected by this [44],[45],[46]. However, by producing beta-lactamase, bacteria can develop resistance to beta-lactam antibiotics. In this investigation, Gram-negative clinical isolates from tertiary care hospitals were simultaneously screened for extended-spectrumbeta-lactamases (ESBL) and ampCbeta-lactamases [47].

**Materials and Methods**

**Materials and equipments:** Peptone water, Simmons citrate water, Urease agar, Triple Sugar iron agar medium, Mannitol motility medium, Mueller Hinton agar plates, Antibiotic discs, Inoculation loop, Incubator, Microscope.

**Study design and clinical isolates:** A total of two hundred aerobic gram negative bacilli were isolated in clinical microbiology laboratory from various clinical samples such as urine, pus, sputum and other body fluids over a period of 6 months (April 2018 to October 2018). The study design was appreciated by Saveetha medical college and hospital, CHENNAI. Each sample was streaked on nutrient agar and mac conkey agar plates and incubated at 37 degree for 24 hours. After incubation, all the gram negative bacilli were identified by routine biochemical tests.

**Test of Extended Spectrum beta lactamase production**

**Double disk synergy test**

Enterobacteriaceae cultures that exhibited intermediate/resistance to third generation cephalosporins were screened to detect the ESBL producers.

The test inoculums (0.5Mc Farland) was spread onto Mueller hinton agar with a sterile cotton swab. Amoxicillin plus clavulanic acid (20 mg+10mg) disc was placed in the centre and the ceftazidime (30mg) and cefatoxime (30mg) disks were placed on either side at a distance of 15mm centre to centre from amoxicillin plus clavulanic disc. Plates were incubated at 35 degree Celsius for 18-20 hrs. and the pattern of zone of inhibition was noted.

Isolates that exhibited a distinct shape/size with potentiation towards amoxicillin +clavulanic disks were considered potential ESBL producers and short listed for confirmation of ESBL producers [48].

**Phenotypic confirmation Test by Disk Diffusion Assay**

ESBL production was confirmed among potential ESBL Producing isolates by phenotypic tests. Sensitivity, disks containing third generation cephalosporin with and without clavulanic acid (10 microgram)and aztreonam (30 microgram), aztreonam and cefatoxime (10 mg). Disk diffusion assay was carried out as per guidelines of NCCLS and differences in zone diameters between disks with and without clavulanic acid were recorded.

Organisms will be considered as ESBL producer if there appears a 5mm increase in zone diameter of ceftazidime/clavulanic disc and that of ceftazidime disc alone.

Escherichia coli ATCC25922 and klebsiella pneumonia strain 48188 will be used as positive and negative controls respectively [48].

**Detection of AmpC Beta Lactamases**

**Modified double disk approximation method**

The test inoculum (0.5Mc Farland turbidity) was spread onto Mueller hinton agar with a sterile cotton swab. Cefoxitin (30 microgram) disc was placed in the centre and the ceftazidime (30 microgram), and cefatoxime (30 microgram) disks were placed on either side at a distance of 20 mm centre to centre from amoxicillin plus clavulanic disc. Plates were incubated at 35 degree Celsius for 18-20 hrs and the pattern of zone of inhibition was noted.

Isolates showing reduced susceptibility to either of the test drugs (ceftazidime or cefotaxime) and cefotixin were considered as presumptive AmpC producers and further confirmed by AmpC disk test [49].

**AmpC Disk Test**

All isolates were simultaneously checked by AmpC disk test. A lawn culture of Escherichia coli ATCC25922 was prepared on MHA plate. Sterile disks (6mm) were moisture with sterile saline (20 ml) and inoculated with the several colonies of test organism. The inoculated disk was then placed beside a cefoxitin disk (almost touching) on the inoculated plate. The plates were incubated overnight at 35 degree Celsius. A positive test appeared as a flattening or indentation of the cefoxitin inhibition zone in the vicinity of the test disk. A negative test had an undistorted zone.

Escherichia coli ATCC25922 and klebsiella pneumoniae strain 48188 will be used as positive and negative controls respectively [49].

**Results and Discussion**

During the study period of seven months from April 2010 to October 2010 various samples such as urine, pas, wound swab, exudates, cereberospinal fluid, pleural fluid, synovial fluid, stool sample, and catheter tips were taken from the inpatients and outpatients attending all departments in Saveetha Medical College and Hospital. A total of two hundred aerobic Gram negative bacilli have been isolated from the above specimens.

Distribution of aerobic Gram negative bacilli isolated from various samples was shown in Table-1.

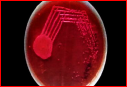
**Table No. 1 Distribution of aerobic gram negative bacilli isolated from various samples.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Organism** | **Urine** | **Pus** | **Sputum** | **Blood** | **Total** |
| **Escherichia Coli** | 103 | 6 | 3 | 0 | 112 |
| **Klebsiella pneumoniae** | 15 | 5 | 8 | 0 | 28 |
| **Pseudomonas aeruginosa** | 0 | 7 | 4 | 0 | 11 |
| **Citrobacter SPP** | 6 | 5 | 0 | 2 | 13 |
| **Enterobacter Cloacae** | 7 | 8 | 0 | 8 | 15 |
| **Proteus SPP** | 2 | 2 | 0 | 8 | 4 |
| **Acinetobacter Baumanni** | 4 | 3 | 1 | 0 | 8 |
| **Non fermentative gram negative bacilli** | 5 | 4 | 0 | 8 | 9 |
| **Total** | 142 | 32 | 16 | 10 | 200 |

**Sensitivity Percentage Distribution of Gram negative Bacilli to Various Antibiotics is shown in Table-2**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Antibiotics** | **E.coli** | **K.pneumonia** | **P.aeruginosa** | **Proteus spp** | **Citrobac. spp** | **A.baumanii** | **E.cloacae** |
| **Imipenem** | **100%** | **100%** | **100%** | **100%** | **100%** | **100%** | **100%** |
| **Ampicilin** | **17.8%** | **17.8%** | **36.4%** | **------** | **30.7%** | **---------** | **20%** |
| **Amikacin** | **80.4%** | **82.2%** | **63.6%** | **50%** | **100%** | **50%** | **100%** |
| **Gentamycin** | **43.7%** | **53.6%** | **81.8%** | **50%** | **69.3%** | **62.5%** | **80%** |
| **Nitrofuran** | **95.5%** | **35.7%** | **-----** | **25%** | **61.5%** | **-----------** | **60%** |
| **Norfloxacin** | **27.6%** | **39.3%** | **-----** | **25%** | **61.5%** | **37%** | **40%** |
| **Cefuroxime** | **16.9%** | **10.7%** | **-----** | **-----** | **23%** | **34.6%** | **20%** |
| **Ceptazimidime** | **17.8%** | **28.5%** | **18.9%** | **-------** | **38.5%** | **25%** | **20%** |
| **Cefotaxime** | **23.2%** | **53.6%** | **-----** | **25%** | **61.5%** | **37.5%** | **40%** |
| **Ciprofloxacin** | **49.1%** | **39.3%** | **81.8%** | **75%** | **46.3%** | **62.5%** | **53.4%** |

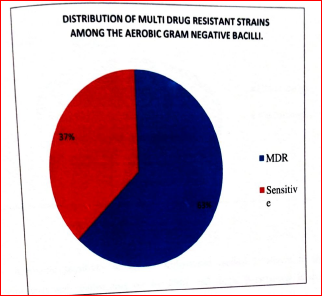
In this study, out of two hundred isolates (200) of gram negative organisms, one twenty six (126) 63% of isolates were resistant to three groups of antibiotics and were moderately sensitive or resistant to three groups of antibiotics and were moderately sensitive or resistant to any of the third generation cephalosporins (3GC- ceftazidime, ceftriaxone, cefotaxime) remaining seventy four (74) of the isolates were sensitive to all antibiotics (37%).

**Mac Conkey Agar with mucoid lactose fermenting colonies of Klebsiella pneumoniae**

**Biochemical test for identification of Escherichia coli.**

## Distribution of multidrug resistant strains among the aerobic gram negative bacilli shown in Figure 1.

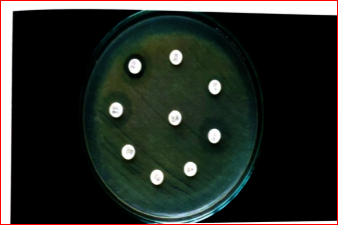
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**Figure 1**

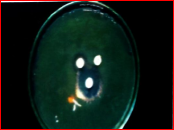
Out of 200 isolates, 126 (63%) muti drug resistant strains have been found. 118 (59%) were ESBL producers of which 54 (27%) were also derepressed mutants (resistant) to cefoxitin and cefotaxime, blunting of the zone towards inducer and increase in zone size by >5 mm), while remaining 64 (32%) were plain ESBL producers. Inducible AmpC beta lactamases production was detected in 3 (1.5%) of the isolates. AmpC mediated beta lactamase production was detected in 3 (1.5%) of the isolates. AmpC mediated beta lactamase was seen in two Klebsiella pneumoniae which are also the ESBL producers and one in Pseudomonas aeruginosa which is a non ESBL producer. Remaining 5 (2.5%) resistant strains were neither ESBL nor AmpC beta lactamase producers.

**Figure -2**

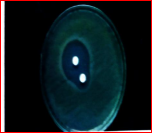
**Antibiotic sensitivity testing by Kirby- Bauer method and screening tests and confirmatory test**

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**Kirby bauer method showing multi drug resistant strains of Escherichia coli**

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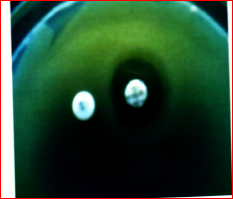
**Screening test for detection of ESBL organism showing enhanced zone between Ca/Ce and Amoxicillin/clavulanic acid (ESBL producer).**

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**Phenotypic confirmatory test (ESBL) organism showing 5 mm increase in zone diameter between Cac and Ca.**

**Screening test and confirmatory test for AmpC beta lactamases is shown in figure 3.**

**Figure-3**

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**Modified double disk approximation method: showing blunting of ceftazidime zone of inhibition adjacent to cefoxitin disc**

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**AmpC disc test: showing flattening of zone of inhibition**

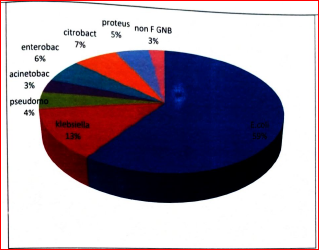
Distribution of ESBL and AmpC beta Lactamase in different aerobic gram negative bacilli is shown in Table-3.

**TABLE No. 3 Distribution of ESBL and AmpC Beta Lactamase in different aerobic gram negative bacilli.**

|  |  |  |  |
| --- | --- | --- | --- |
| **Sl. No.** | **Organism** | **Extended spectrum beta lactamase (ESBL)** | **AmpC spectrum beta lactamase (AmpC)** |
| **1** | **Escherichia coli** | **70** | **0** |
| **2** | **Klebsiella pneumonia** | **15** | **2** |
| **3** | **Pseudomonas aeruginosa** | **5** | **1** |
| **4** | **Acinetobacter baumannii** | **4** | **0** |
| **5** | **Enterobacter cloacae** | **7** | **0** |
| **6** | **Citrobacter spp** | **8** | **0** |
| **7** | **Proteus spp** | **6** | **0** |
| **8** | **Non fermentative gram negative bacilli** | **3** | **0** |
|  | **Total** | **118** | **3** |

**Majority of ESBL producers were Escherichia coli (59%) and klebsiella pneumonia (13%) followed by Citrobacter spp (7%), Enterobacter cloacae (6%), Proteus mirabilis (5%), Pseudomonas aeruginoosa (4%), Acinetobacter baumannii (3%) and non fermentative gram negative bacilli (3%). Distribution of ESBL in different organisms is shown in figure 8.**

**Distribution of ESBL in different organisms**

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AmpC mediated beta lactamase was seen in two (1%) Klebsiella Pneumoniae which are also found to be an ESBL producers and one Pseudomonas Aeruginosa (0.5%) Which is a non ESBL producer.

**Discussion**

Aerobic gram negative bacilli are responsible for numerous diseases. The emergence of multidrug resistance among the aerobic gram negative bacilli has been a major problem in clinical isolates. The most common cause of bacterial resistance to beta lactam antibiotics is the production of beta lactamases. Many of the second and third generation cephalosporins and penicillins were specifically designed to resist the hydrolytic action of major beta lactamases. The latest in the arsenal of these enzymes has been the evolution of extended spectrum beta lactamases. These enzymes are commonly produced by many members of enterobacteriaceae, especially Escherichia coli and klebsiella pneumoniae and efficiently hydrolyze oxyimino cephalosporins conferring resistance to third generation cephalosporins such as cefotaxime, ceftazidime, ceftriaxome, and monobactams such as aztreonam. AmpC beta lactamases are commonly isolated from extended spectrum cephalosporin resistant gram negative bacteria.

Increasing resistance to third generation cephalosporins has become a cause for concern especially among enterobacteriaceae that cause nosocomial infections. The prevalence of extended spectrum beta lactamases among members of enterobacteriaceae constitutes a serious threat to current beta lactam therapy leading to treatment failure and consequent escalation of costs.

In present study out of two hundred (200) isolates, one twenty six (126), multidrug resistant strains have been found (63%). One hundred eighteen (118) were ESBL producers (59%) of which fifty four (27%) were also derepressed mutants, while remaining sixty four (32%) were plain ESBL producers and three (1.5%) were the AmpC beta lactamase producers.

In a similar study done by Rodrigues et al., on detection of lactamases, one fifty one were ESBL producers (53%) which is almost similar as compared to present study. Out of 53% ESBL producers 131 (45.8%) were also derepressed mutants remaining 20 (7%) were plain ESBL producers. Inducible AmpC beta lactamase production was detected in 19 (7%) of the isolates, which is high as compared to our study.

Most of the studies with respect to prevalence of ESBLs among the gram negative bacilli Escherichia coli are the predominant one, followed by Klebsiella pneumonia which is almost similar to other study done by Rodrigues et al. where also Escherichia coli is the predominant ESBL producers (53.6%) followed by klebsiella pneumoniae (19.2%). The AmpC beta lactamases in their study were found to be 7% which is quite high as compared to our study.

Various Indian studies which have the different percentage of ESBL and AmpC beta lactamases among the aerobic gram negative bacilli in shownh in table 4.

|  |  |  |
| --- | --- | --- |
| **Study done and year** | **Percentage of ESBL Isolates** | **Percentage of AmpC beta lactamase isolates** |
| C.Rodrigues et al 2004 | 53% | 7% |
| S.Singhal et al 2005 | 64% | 8% |
| V. Hemalatha et al 2007 | 45% | 9.2% |
| Present study | 59% | 1.5% |

Over two decades, extended spectrum beta lactamases are a rapidly evolving group of beta lactamases and there have been sporadic reports of ESBLs from major hospitals in india and some of them have recorded the incidence to be as high as 60-68%. In our study also we observed almost similar findings regarding the prevalence of ESBL in gram negative organism that is 59%. In present study, analysis of the one hundred and eighteen confirmed ESBL isolates revealed that ESBLs were predominantly present among Escherichia coli (59%), followed by klebsiella pneumoniae (13%) and other enterobacteriaceae such as Proteus mirabilis and Proteus vulguris 9% , Citrobacter diversusand Citrobacter fruendii 7%, Enterobacter cloacae 6%, Pseudomonas aeruginosa 4%, Acinebacter baunmannii 3%.

All the ESBL and AmpC producing and chromosomal mediated and moreover they are found to be multi drug resistant organisms. In present study all the strains were sensitive to imipenem and Amikacin. Among the non beta lactum antibiotics second most effective drug in Ciproflooxacin and its sensitivity varies between 58.2% to 62.98%. Sensitivity to Nitrofurantoin, Cefotaxime and Norfloxacin are 36.67%, 34.4% and 32.2% respectively and Ampicillin and Cefuroxime are 17.53% and 15.03% sensitive to all strains respectively.

**Conclusion**

Out of two hundred isolates of gram negative organisms 63% of the isolates were multi drug resistant strains and remaining 37% of the isolates were sensitive to all antibiotics. Antibiogram of all gram negative bacteria showed 100% sensitivity to Imipenem and Amikacin followed by Ciprofloxacin (62.98%), Nitrofurantoin (36.67%), Cefotaxime (34.40%) and Norfloxacin (32.96%), Ampicillin (17.53%), and Cefuroxime (15.03%). The predominant AmpC beta Lactamase producers among the aerobic Gram dnegative bacilli is Klebsiella pneumonia (1%) which is also a ESBL producer followed by Pseudomonas aeruginosa (0.5%) which is a non ESBL producer. Ideal empirical treatment for gram negative bacilli are Imipenem and Amikacin.

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