

INVESTIGATION OF THE PHENOTYPES OF AEROBIC GRAM-NEGATIVE BACTERIA WITH EMPHASIS ON AmpC β -LACTAMASES AND ESBL

Abstract

β -lactamases are a type of β -lactam resistance that is most common. Expanded spectrum β -lactamases (ESBLs), which are now being identified in large numbers all over the world, are an important category of β -lactamases along with inducible AmpC β -lactamases and derepressed mutants. The goal of the current study was to precisely analyze beta-lactamase production in medical isolates, ESBLs, and AmpC β -lactamases, by reordering the traditional discs employed in reporting susceptibility. *Klebsiella pneumoniae* and *Escherichia coli* are the two most prevalent bacteria identified in mid stream urine (MSU) samples from all across the world. The primary producers of the extended spectrum β -lactamase, which significantly limits the therapeutic treatment of urinary tract infections, are these uropathogens. A rising problem in the world is bacterial strains that are resistant to antibiotics. Urinary tract infections (UTIs) rank among the most common bacterial diseases in humans in both the community and hospital settings. *Klebsiella pneumoniae* and *Escherichia coli* are the two pathogens found in urine that are most frequently seen. Gram-negative microbes continue to gain resistance to β -lactam antibiotic mostly due to β -lactamases. In this study, screening tests and confirmatory tests were conducted along with antibiotic sensitivity testing using the Kirby-Bauer method. Confirmatory test and Screening test for AmpC beta was also done.

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

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I. INTRODUCTION

The Gram-negative bacteria that are aerobic majority of bacterial discovered variants 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]. The metabolic pathways used by non-fermentative bacterial species to break down carbohydrates do not include 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]. The main cause of gram-negative microorganisms developing resistance to beta lactam medicines is beta lactamases. Early in the 1980s, cephalosporins were utilized for the first time in clinical settings to fight bacteria that had developed antibiotic resistance brought on by beta-lactamases [13], [14], [15]. This was heralded as a breakthrough in the fight over germs that are resistant to medicines because of β -lactamases [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 development of additional Gram-negative bacteria resistant 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. Recent publications have found bacteria that produce ESBL with an unusually broad spectrum of antibiotic resistance [26],[27],[28],[29]. The bacterial enzymes known as betalactamases deactivate beta lactam antibiotics through hydrolysis, producing inactive molecules [30], [31],[32]. Some bacteria develop beta-lactamases, which make them resistant to β lactam antibiotics like, Cephalosporin, Cephamycin, Penicillin and Carbapenems [33]. These antibiotics all share the same component in common with one [34],[35]. In view of the high prevalence of beta-lactamase production, prompt action is required on the point of infection control and therapeutic perspective 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 bacterial resistance in gram-negative species 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 synthesizing 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, Isolates from clinical trials with a gram-negative organism from higher care facilities were simultaneously screened for extended-spectrum beta-lactamases (ESBL) and ampC beta- lactamases [47].

II. MATERIALS AND METHODS

- 1. Materials and Equipments:** Peptone water, Simmons citrate water, Urease agar, Triple Sugar iron agar medium, Mueller Hinton agar plates, Mannitol motility medium, Antibiotic discs, Inoculation loop, Incubator, Microscope.
- 2. Study Design and Clinical Isolates:** Over the course of six months (April 2018 to October 2018), 200 aerobic gram-negative bacilli were identified in a clinical microbiology lab from a variety of clinical samples including urine, sputum, pus, and other body fluids. The Saveetha medical college and hospital in Chennai expressed appreciation for the study's design. On both MacConkey agar and nutritional agar plates, each sample was streaked, and it was then left to sit for 24 hours at 37 degrees. Following incubation, regular biochemical assays allowed for the identification of all the gram-negative bacilli.
- 3. Development of Extended Spectrum Beta Lactamase test Test of Double Disk Synergy:** The creators of ESBL were found by screening Enterobacteriaceae cells which showed moderate or resistant properties to third generation cephalosporins.

Using a sterilized cotton swab, the test inoculums (0.5 McFarland) were applied to Mueller hinton agar. The ceftazidime (30mg) and cefatoxime (30mg) disks were positioned on either side of the amoxicillin plus clavulanic acid (20mg+10mg) disc at an angle of 15 mm center to center relative to the amoxicillin plus clavulanic acid disc. Samples were left to incubate for 18–20 hours at 35 degrees Celsius to identify the area of resistance pattern.

Likely ESBL producers were identified and selected for verification as ESBL producers among isolates which displayed a distinctive shape and size with potency toward amoxicillin+clavulanic disks [48].

- 4. Study for Phenotypic Verification Using Diffusion of disks:** Phenotypic assays amongst samples that might produce ESBLs verified ESBL formation. Sensitivity disks with a third-generation cephalosporin, aztreonam (30 micrograms), clavulanic acid (10 micrograms), and cefatoxime (10 milligrams) are available. Following NCCLS guidelines, a disk diffusing assay was conducted, and changes in zone widths between disks containing and excluding clavulanic acid were noted.

If the diameter of the zone of the ceftazidime/clavulanic disc increases by 5 mm compared to the zone width of the ceftazidime disc by itself, the living thing will be regarded to be an ESBL generator.

As negative and positive controls, accordingly, *Klebsiella pneumonia* strain and *Escherichia coli* ATCC25922 48188 will be utilized [48].

III. DETECTION OF AMPC BETA LACTAMASES

- 1. Altered Double Disk Estimation Technique:** Using a sterile cotton swab, the experiment an inoculum (0.5 McFarland turbid) was applied to Mueller hinton agar. Ceftazidime (30 microgram), cefatoxime (30 microgram), and cefoxitin (30 microgram) disks have been placed on both sides of the amoxicillin plus clavulanic discs at an offset of 20 mm center to center from each other. Plates were incubated for 18 to 20 hours at 35

degrees Celsius to identify the region of inhibition patterns. Ceftazidime or cefotaxime-resistant isolates and cefotixin-resistant isolates were assessed to be potential AmpC growers, and the latter was validated by an AmpC disks analysis [49]. Testing for AmpC Disc: AmpC disk testing was used in order to verify the isolates. *Escherichia coli* ATCC25922 grass culture was established on an MHA plate. Several colonies of the test organism were injected onto sterile disks (6mm) that had been moistened using sterile saline (20 ml). On the inoculated plate, the inoculated disk was next to a ceftaxitin disk, practically touching. The dishes were left to incubate at 35 degrees Celsius for the entire night. A flattening or indentation of the ceftaxitin inhibition zone close to the test disk indicated a successful test. The zone was undistorted in a negative test. As positive and negative controls, accordingly, *Klebsiella pneumoniae* strain 48188 and *Escherichia coli* ATCC25922 will be utilized [49].

IV. RESULTS AND DISCUSSION

Different specimens were collected over the course of the seven-month investigation from April 2018 to October 2018 such as urine, pus, wound swab, exudates, cerebrospinal 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. From the aforementioned samples, 200 aerobic Gram negative bacilli have been identified. Table 1 displays the abundance of aerobic Gram negative bacilli that have been collected from diverse collections.

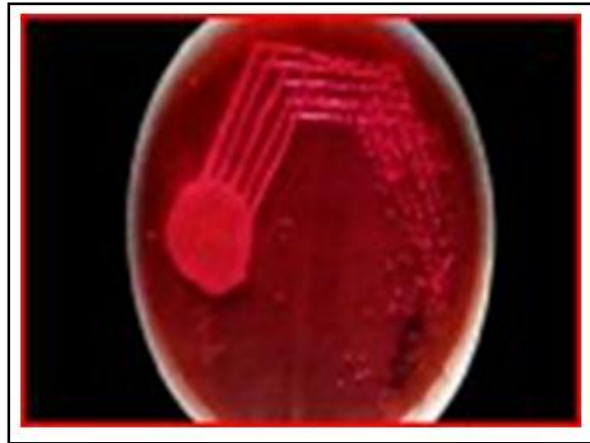
Table No. 1 Distribution of aerobic gram negative Bacilli Isolated from Various Samples.

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

Ratio of sensitivities Table 2 displays how Gram negative Bacilli respond to various antibiotics.

Antibiotics	E.coli	Klebsiella pneumonia	Pseudomonas aeruginosa	Proteus spp	Citrobacter. spp	Acinetobacter. baumannii	Enterobacter. 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%
Nitrofurantoin	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%
Ceptazidime	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 have been moderately susceptible to three classes of antibiotics and sensitive or opposed to three groups of antibiotics and were moderately sensitive or resistant With any third- generation cephalosporin antibiotic (3GC- ceftazidime, ceftriaxone, cefotaxime) remaining seventy four (74) of the isolates were sensitive to all antibiotics (37%).

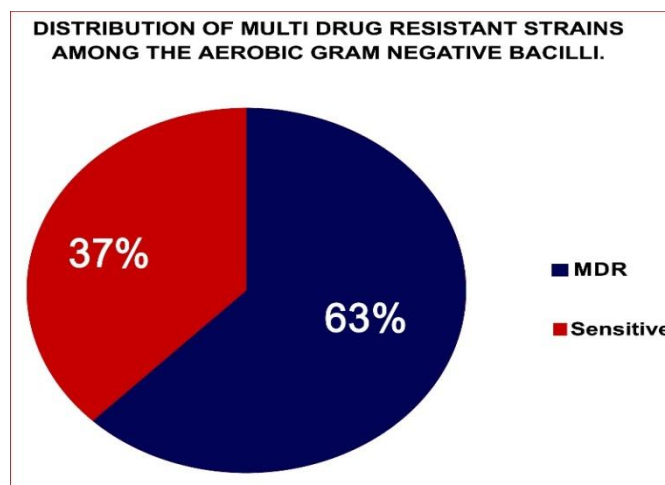


Mac Conkey Agar with mucoid lactose fermenting colonies of *Klebsiella pneumoniae*



Biochemical test for identification of *Escherichia coli*

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



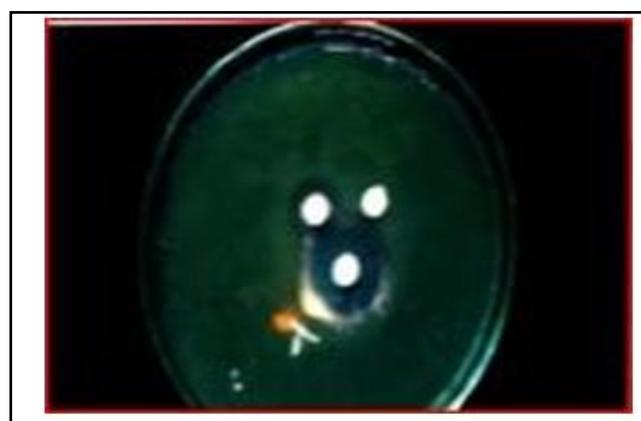
200 isolates were discovered, and 126 (63%) of those were multidrug resistant strains. A total of 118 people (59%) produced ESBLs, of which 54 (27%) additionally generated derepressed mutants (resistant to ceftiofuran and ceftazidime, blunting the zone toward inducer, and increasing the zone width by >5 mm), and the remaining 64 (32%) were simple ESBL producers. Three of these isolates, or 1.5% of them, produced induced AmpC beta lactamases. In 3 (1.5%) of the isolates, ampC-mediated beta lactamase synthesis was found. 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.

Kirby-Bauer technique of determining sensitivity to antibiotics tests, including screening tests and confirmatory tests

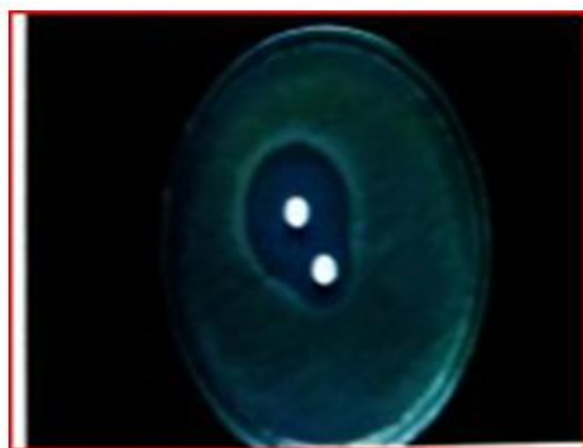
Kirby Bauer method showing multi drug resistant strains of *Escherichia coli*



Figure-2



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



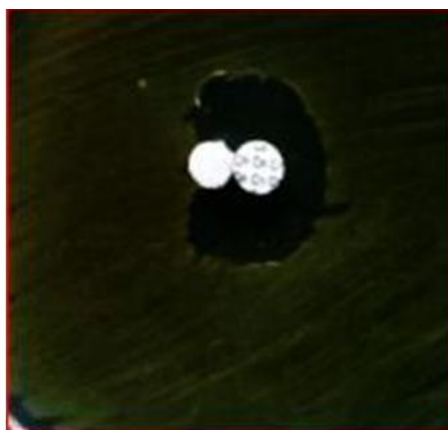
Zone width between Cac and Ca increased by 5mm in phenotypic confirmatory test (ESBL) organism

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



Figure-3

Modified double disk approximation method: ceftazidime inhibitory zone blunting next to ceftoxitin discs



AmpC disc test: displaying flattening of zone of inhibition

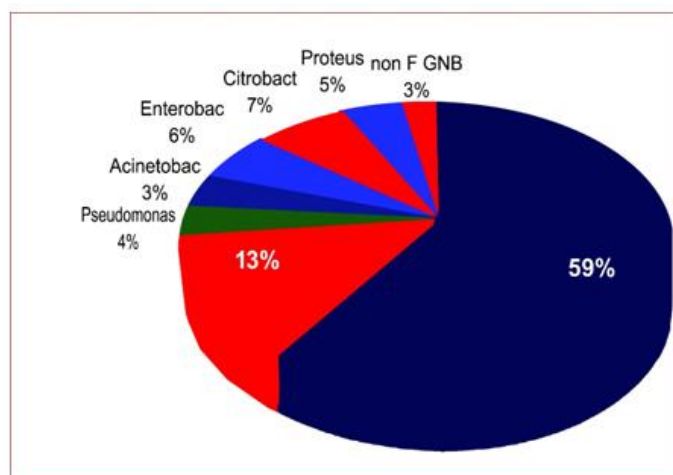
Table 3 displays the abundance of AmpC beta and ESBL Lactamase in several aerobic gram negative bacilli.

Table 3: AmpC and ESBL Beta Lactamase distribution in several aerobic gram-negative bacteria.

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 pneumoniae (13%) followed by Citrobacter spp (7%), Enterobacter cloacae (6%), Proteus mirabilis (5%), Pseudomonas aeruginosa (4%), Acinetobacter baumannii (3%) and nonfermentative gram negative bacilli (3%). The abundance of ESBL indifferent microbes is displayed in figure 8.

Distribution of ESBL in Different Organisms



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

V. DISCUSSION

Multiple illnesses are brought on by aerobic gram-negative bacteria. A significant issue with isolates from patients has been the growth of multidrug susceptibility within aerobic gram-negative microbes. The development of beta lactamases is the most frequent reason for microbial resistance to the beta lactam medicines [50]. A number of both the second and third generations cephalosporins and penicillins were created expressly to withstand the hydrolytic effects of powerful beta lactamases. Extended spectrum beta lactamases are the newest addition to this group of enzymes. The ability to effectively hydrolyze oxyimino cephalosporins confers resistance to third generation cephalosporins like ceftazidime, cefotaxime, monobactams and ceftriaxome, like aztreonam. These enzymes are frequently generated by numerous species of the enterobacteriaceae, particularly *Escherichia coli* and *Klebsiella pneumoniae* [51]. Extensively spectral cephalosporin resistant gram negative bacteria are frequently used to isolate ampC beta lactamases.

Particularly within enterobacteriaceae responsible for nosocomial infections, rising third generation cephalosporin resistance has become a source of worry. The predominance of wider spectrum beta lactamases amongst enterobacteriaceae members poses a severe danger to the efficacy of present beta lactam treatment, and which will result in an increase in prices [51].

Sixty-three percent (63%) of the 200 isolates in the current investigation were determined to be multidrug resistant pathogens. One hundred eighteen (118) were the producers of ESBL (59%) out of which fifty-four (27%) also consisted of derepressed mutants, while sixty-four (32%) were plain ESBL providers and three (1.5%) were AmpC β lactamase producers.

Similarly to the current investigation, Rodrigues et al.'s research on the detection of lactamases found that 150 of the lactamases detected were makers of ESBL (53%). The other 20 (7%) were plain ESBL growers, leaving 131(45.8%) of the 53% producers of ESBL who were also derepressed mutants. 19(7%) of the isolates had inducible AmpC beta lactamase production, which is higher than expected based on the results of our investigation [52].

Escherichia coli has been the most prevalent producer of ESBLs (53.6%) in the majority of studies on the prevalence of ESBLs between gram-negative bacilli, ahead of *Klebsiella pneumoniae* (19.2%). This finding is almost identical to that of another study by Rodriguez et al. In comparison to our analysis, their study reported an AmpC beta lactamase concentration of 7%, which is rather high.

Various Indian studies which have the different percentage of AmpC and ESBL beta lactamases part of the aerobic gram negative bacilli in shown in table 4 [52], [53],[50].

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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%

Some are sporadic observations regarding wider spectrum beta lactamases from important hospitals in India, and a few of these cases indicate the incidence to be as much as 60–68% [51]. During the past 20 years, this group of beta lactamases has undergone fast evolution. In our study also we observed almost similar findings regarding the prevalence of ESBL in gram negative organism that is 59%. In the current investigation, evaluation of the 118 confirmed ESBL samples showed that ESBLs appeared most frequently found in *Escherichia coli* (59%), followed by *Klebsiella pneumoniae* (13%) and other enterobacteriaceae such as *Proteus mirabilis* and *Proteus vulgaris* 9% , *Citrobacter diversus* and *Citrobacter freundii* 7%, *Enterobacter cloacae* 6%, *Pseudomonas aeruginosa* 4%, *Acinetobacter baumannii* 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 Ciprofloxacin 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.

VI. CONCLUSION

Sixty-three percent of the 200 isolates of gram-negative bacteria were multidrug resistant strains, where as the remaining 37percent were antibiotic sensitive. Imipenem and Amikacin were the only antibiotics that were 100% effective against all gram-negative bacteria, preceding 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 negative bacilli is *Klebsiella pneumoniae* (1%) which is also an ESBL producer followed by *Pseudomonas aeruginosa* (0.5%) which is a non ESBL producer. Ideal empirical treatment for gram negative bacilli is Imipenem and Amikacin.

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