Life Science Informatics Publications

Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences

Journal Home page http://www.rjlbpcs.com/

RJLBPCS

ISSN 2454-6348

Original Research Article **DOI: 10.26479/2018.0403.38**

CO EXPRESSION OF BETALACTAMASES IN GRAM NEGATIVE *BACILLI* ISOLATED FROM CLINICAL SAMPLES

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ABSTRACT: The newer beta β-lactamases have emerged as a cause of antimicrobial resistance in gram negative bacteria. In India the prevalence ranges from 6.6% to 68%. The coexistence of different classes of β-lactamases in a single bacterial isolate may pose diagnostic and treatment challenges. The AmpC producing organisms can act as a hidden reservoir for the ESBLs. Also, the high-level expression of the AmpC β-lactamases may mask the recognition of the ESBLs and it may result in a fatal and an inappropriate antimicrobial therapy. This study was conducted to detect co-expression of all three of these newer betalactamase in gram negative bacilli. *Klebsiella spp* was the commonest isolate (28.47%) followed by *E.coli* (26.48%), Other isolates were *Pseudomonas aeruginosa* (19.54%), *Enterobacter spp* (8.92%), *Acinetobacter spp* (8.92%) and *Citrobacter spp* (7.64%). ESBL production was seen more in *E.coli* followed by *Klebsiella* spp and *pseudomonas aeruginosa*. AmpC production was seen more in *Acinetobacter spp*. MBL Production was seen more in *E.coli.* Co-expression of newer β lactamases like ESBL, AmpC, MBL were found to be more in *Acinetobacter spp* and *Enterobacter spp.* The high prevalence of these organisms in the ICUs emphasizes the need for an early detection of the β-lactamase producing organisms by simple screening methods, which can help in providing an appropriate antimicrobial therapy and in avoiding the development and the dissemination of these multidrug resistant strains.

KEYWORDS: Gram negative bacteria, Coexpression, ESBL, AmpC, MBL, Gram negative bacteria

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1.INTRODUCTION

The most common cause of bacterial resistance to betalactam antibiotics is the production of betalactamase. The newer beta βlactamases namely extended spectrum β-lactamases, AmpC β-lactamases and Metallo- βlactamases have emerged as a cause of antimicrobial resistance in gram negative bacteria[1]. Genes for all these three enzymes are often carried on plasmids facilitating rapid spread between microorganisms[1]. The incidence of these betalactamase ranges from 1.8% to 74% worldwide[2] and in India the prevalence ranges from 6.6% to 68%[3].These enzymes are often co expressed in the same isolate. The presence of ESBL and AmpC β lactamases in a single isolate reduces the effectiveness of βlactam- βlactamase inhibitor combination while Metallo-β lactamases confer resistance to Carbapenems. This study was conducted to detect co-expression of all three of these newer betalactamase in gram negative bacilli. Since the prevalence of *Enterobacteriaceae* producing ESBLs, AmpC, and MBL is being increased, it is mandatory that the routine clinical microbiology laboratory must employ detection methods for these enzymes, which are sensitive enough to recognize the level of resistance that would be achieved by the situation given in vivo. In the years since these enzymes were first described, a number of different testing methods have been suggested.

AIMS AND OBJECTIVES:

a) Antibiotic sensitivity testing for gram negative bacterial isolates.
b) Screening of isolates for co expression of Extended Spectrum β-Lactamase, AmpC β-lactamase and Metallo β-lactamase production

2. MATERIALS AND METHODS

Seven hundred and six isolates from various clinical samples from Kamineni institute of medical sciences Hospital, Narketpally, both from out-patients and in-patients, were processed during the period of 2010 to 2012. Clinical samples mainly included were urine (298), sputum (202), blood (24), pus (98), Endotracheal Tube (54) and body fluids (30). 706 gram negative bacilli isolated from various clinical samples like blood, pus, urine, sputum, body fluids were included in the study. All the clinical isolates other than gram negative bacilli were excluded from the study. The antibiotic sensitivity test was performed by modified Kirby Bauer disc diffusion technique with commercially available HiMedia discs according to CLSI guidelines on Mueller Hinton agar plates. In the present study susceptibility was tested against antibiotics mentioned below[4]. The strength of antibiotic discs used (Discs obtained from Himedia Laboratories Pvt. Ltd. Mumbai.)[5]

Piperacillin 30µg	Cephoxitin 30µg
Gentamicin10µg	Ceftazidime 30µg
Amikacin 30µg	Cephotaxime 30µg
Ciprofloxacin 5µg	Imipenem 10µg

Nitrofurantoin 300 µg	Piperacillin / Tazobactum 100/10µg
Norfloxacin 10 µg	Amoxicillin / Clavulanic acid 20/10µg
Cotrimoxazole 1.25/23.75 µg (Trimethoprim-sulphomethoxazole)	

The zone of inhibition was measured and interpreted according to CLSI criteria.

At least three to five well isolated colonies of the isolate of the same morphological type were selected from Nutrient agar plate culture. The top of each colony is touched with a wire loop and the growth transferred to a tube containing 5ml of Nutrient broth. The broth culture was incubated at 37ºC for 2-4hrs till turbidity is equal to or 0.5 McFarland standards.

ESBL Detection

Gram negative isolates resistant to one of the 3rd generation cephalosporins were subjected to ESBL detection. ESBL detection was carried but by two procedures. Demonstration of Synergistic action between a 3rd generation Cephalosporin test antibiotic and Augmentin disc (20µg amoxycillin + 10µg clavulanic acid) by Double Disc Synergy Test (DDST). DDST positive strains were further confirmed by Phenotypic Confirmatory Disc Diffusion Test PCDDT) using a 3rd generation Cephalosporins alone and in combination clavulanic acid (30µg).

Double Disc Synergy test[6] (DDST)

In DDST synergy were determined between a disc of Augmentin and 30µg disc of 3rd generation Cephalosporin test antibiotic. The standardized 0.5 McFarland inoculum of gram negative bacilli was swabbed on to a Mueller Hinton agar plate by lawn method. A disc of Augmentin was placed in the center and the 3rd generation Cephalosporin i.e. Ceftazidime, Cefotaxime and Ceftriaxone, discs were placed 15mm apart from the central Augmentin disc. MHA plate was incubated overnight at 37˚C.

The strains were considered ESBL producer if they satisfied the below mentioned criteria.

- Inhibition zone around the test antibiotic showed a clear extension towards Augmentin disc.
- If neither disc was inhibitory alone but bacterial growth inhibited between two discs.
- Broadening of the inhibitory zone of 3rd generation cephalosporin towards the Augmentin disc.

Phenotypic Confirmatory Disc Diffusion Test [6] (PCDDT)

Both Cephotaxime (30µg) and Ceftazidime (30µg) disc alone and in combination with clavulanic acid (30µg) were used in this test. While performing antibiotic testing ceftazidime 30µg and ceftazidime 30µg plus clavulanic acid (30µg/ 10µl) were placed on MHA plate, these MHA plates after overnight incubation at 37^0C were interpreted as follows. An increase in zone diameter of ≥ 5 mm for ceftazidime, tested in combination *K. pneumoniae* with clavulanic acid versus its zone when tested alone was considered as ESBL producer. *Klebsiella pneumoniae* ATCC 700603 (ESBL positive) was used as quality control for ESBL test. In PCDDT *Klebsiella pneumoniae* ATCC

700603 shows> 5mm increase in ceftazidime/ clavulanic acid zone diameter.

AmpC Betalactamase detection

Gram negative isolates that yielded a cefoxitin zone diameter less than 18 mm and resistant to 3rd generation cephalosporins (screen positive) were tested for AmpC enzyme production by AmpC disc test.

AmpC Disc test [7]

A lawn culture of *E.coli* is prepared on Mueller Hinton agar plate. Sterile disc (6mm) is moistened with sterile saline (20 µl) and inoculated with several colonies of test organism. Inoculated disc is then placed beside a Cefoxitin 30µg disc on the inoculated plate.The plate was incubated overnight at 35 ^{0}C. Flattening of the Cefoxitin inhibition zone in the vicinity of test disc indicates positive and Undistorted zone negative.

Metallo-Betalactamase detection[8]

Gram negative bacilli were tested for MBL production by Imipenem-EDTA combined disc test. Organism was inoculated on to Mueller-Hinton agar as lawn culture. Two 10 µg Imipenem discs were placed at 20mm centre to centre on the plate. 10 µl of 0.5M EDTA (750 µg) solution was added to one of the Imipenem disc and incubated overnight. Enhancement of zone of inhibition of Imipenem + EDTA disc compared to that of Imipenem disc alone by ≥ 7mm was considered positive for MBL production.

3. RESULTS AND DISCUSSION

706 gram negative consecutive, non repetitive clinical isolates were studied for co expression of ESBL, Amp C and MBL production in Gram negative bacilli. Most of the samples were from inpatients belonging to high risk areas like ICU, NICU, labour room, post operative ward (Fig-1). Out of 706 isolates, commonest isolates were *Klebsiella spp* 201 (28.47%), *E.coli*187 (26.48%) and *P.aeruginosa* 138 (19.54%) (Fig-3). ESBL detection rate was found more by PCDDT. DDST has missed 5 cases of ESBL production in *E.coli* and *Klebsiella*, 3 cases in *Pseudomonas,* 2 each in *Citrobacter, Enterobacter* and *Acinetobacter* respectively. (Fig-6). Majority of AmpC producers were *Acinetobacter, (17.46%) Pseudomonas (11.59%.)(*Fig-7)**.**Majority of MBL producers were *E.coli* (19.62%) and *Acinetobacter* (19.46)(Fig-8)**.** Co-expression of all the three βlactamases was found more in *Acinetobacter spp (9.52) Enterobacter spp* (9.52),(Table-3). The most common cause of bacterial resistance to betalactam antibiotics is the production of betalactamases. ESBLs represent a major group of betalactamases currently being identified worldwide in large numbers along with inducible AmpC betalactamases and derepressed mutants. In recent years, MBL genes have spread from *P.aeruginosa* to members of *Enterobacteriaceae.* These enzymes are plasmid mediated and multidrug resistance is a characteristic feature of strains producing these enzymes. The overall prevalence of ESBL, AmpC, MBL producers are found to vary greatly in different geographical

areas and in different institutes. In the present study, an attempt has been made to know the prevalence of ESBL, AmpC, and MBL in the gram negative bacilli and their antibacterial susceptibility pattern. Out of 706 isolates screened 38.52% were ESBL, 10.33% were inducible AmpC and 9.20% were MBL producers. The most common isolate was *Klebsiella* spp. (28.47%); similar to studies of Ratna et al [9] (34.4%) and shukla et al[6] (30.14%), whereas study conducted by Kumar MS et al[4] *E.coli* (50.29%) was the most common isolate. 26.48% *E.coli* isolated which is similar to the studies conducted by Luzzaro F et al [10] (31.9%) and Sridhar Rao et al [11](35.78%). *Pseudomonas aeruginosa* was isolated in 19.54% isolates which is similar to the studies conducted by Franklin et al [12] (25.7%). *Enterobacter* spp accounted for (8.92%) of isolates, similar to the study of Menon T et al [13] (10%); Luzzaro F et al [10] (7.5%). *Acinetobacter* spp accounted for (8.92%) isolates are less compared to the study conducted by Franklin et al [12] (8.8%). *Citrobacter* spp accounted for (2.5%) isolates, similar to the study of Ratna et al [9] (3.8%). (Table-10)

Percentage of ESBL production

In the present study 38.52% isolates were ESBL producers similar to studies by Neelam Taneja et al [14] (36.5) and Shukla et al [6](30.18). In the present study 10.33% isolates produce inducible AmpC betalactamases, similar to studies of Rodrigues et al [15] (7%), less compared to the study of Singhal P et al [7] (24%). This shows that the chromosomally encoded AmpC betalactamases are prevalent in our setting. In the present study 48.66% of *E.coli* were ESBL producers which is similar to studies done by Loveena et al [16] but less than the studies done by S.S.Chatterjee et al[1] (81.80)and Rajini et al [17](72.00%).6.96% of *Klebsiella* were AmpC producers is similar to the study done by Loveena et al[16]. 19.6% of *E.coli* were MBL producers which is similar to the study done by loveena et al[16].

Comparison of co-expression of β lactasmases producing GNB from various studies.

The ESBL and AmpC co production was detected in 9.77% of the isolates in the present study, which was in concordance with the studies done by Parul sinha et al [18]. (8%) and Loveena et al [17] (6.59%). ESBL and MBL co-production was detected in 4.81% of the isolates in the present study which is slightly higher than the studies done by Mendiratta et al [19] (8.62%) and Loveena et al [16](8.79%). AmpC and MBL co production was detected in 6.23% of the isolates in the present study which is lesser than the study done by Loveena et al[1](3.67%). ESBL+AmpC+MBL was seen in 5.09% which is lesser than the studies done by S.S.chatterjee et al [1] (23.70) and Loveena et al [16](19.04). β lactamase production in the present study was lower compared to other studies. This may be due to the following reasons-

i. Other studies were done in higher tertiary care centres (urban centres), where as the present study was done in a rural medical college hospital.

ii. Isolated organisms were mostly from the hospital acquired infections in other studies where as in the present study they were from both inpatients and outpatients.

iii. Sample size and duration in other studies done were of smaller sample size and done over a short duration of time.

Resistance pattern of third generation cephalosporins in the present study was 72.15% which is slightly lesser than the studies done by S Baby Padmini et al [20] (87%) and Priya Dutta et al[21] (86%).

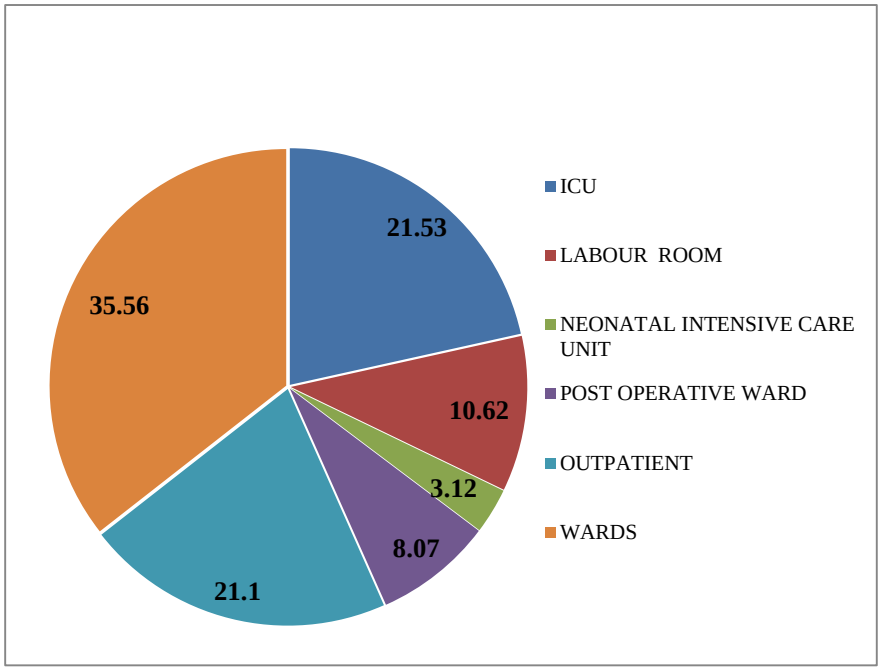

Figure 1: Area wise distribution of samples.

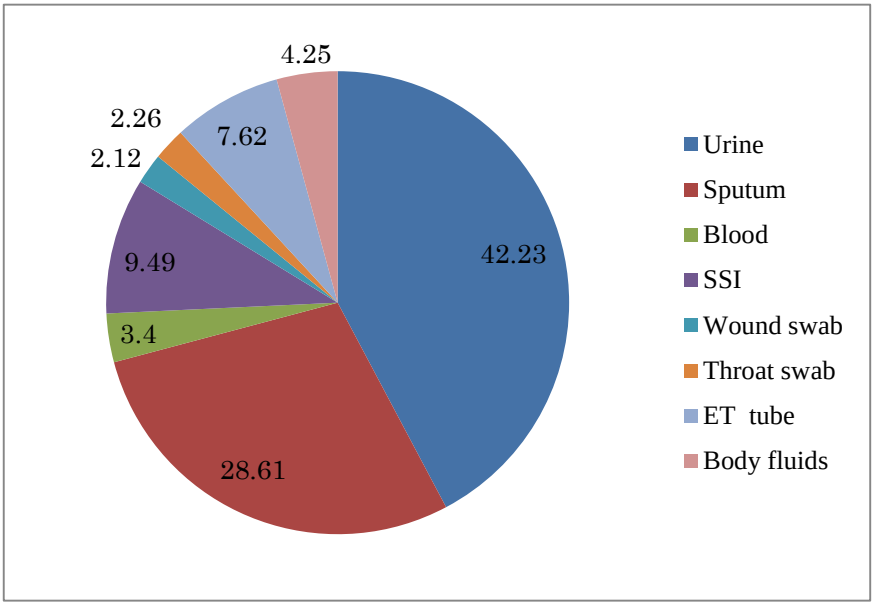

Figure 2: Sample distribution

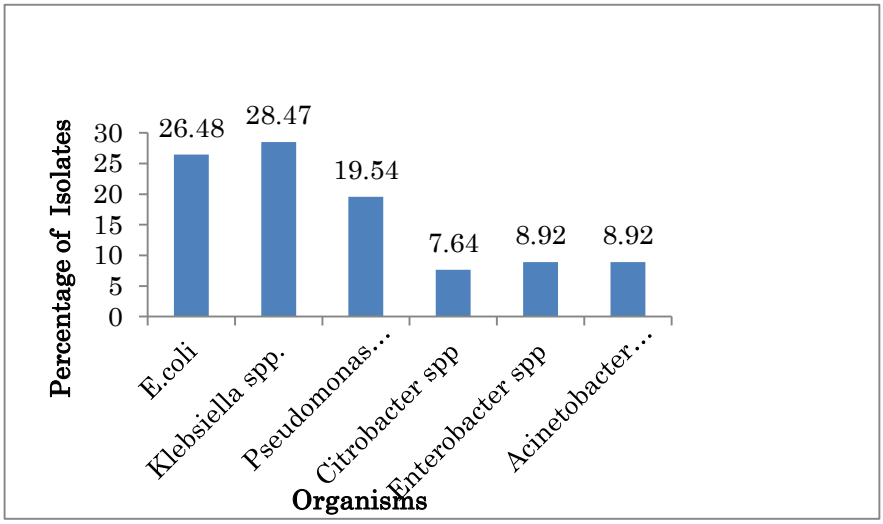

Figure 3: Percentage distribution of organisms

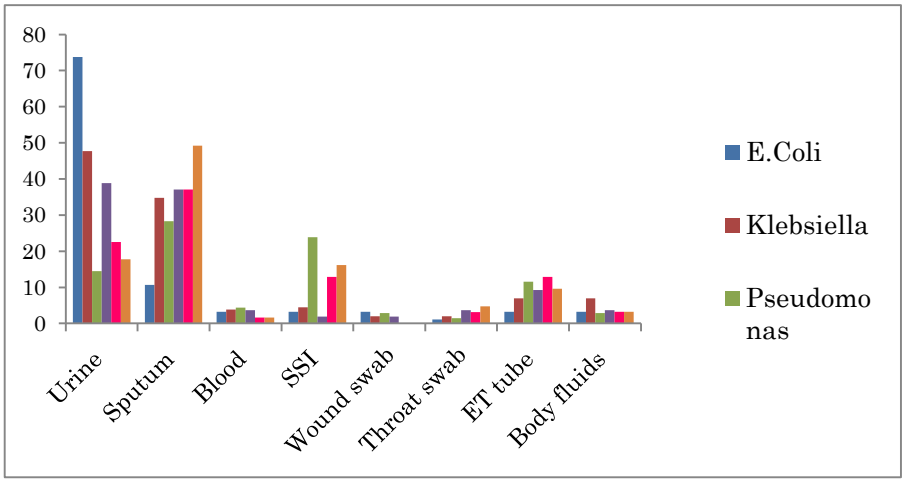

Figure 4: Distribution of isolates in various samples

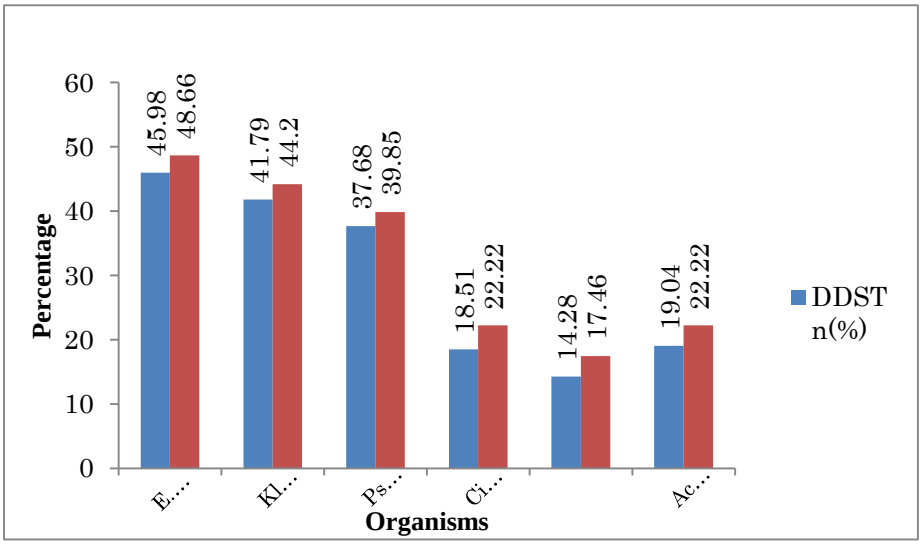

Figure 5: Comparison of DDST and PCDDT in detection of ESBL

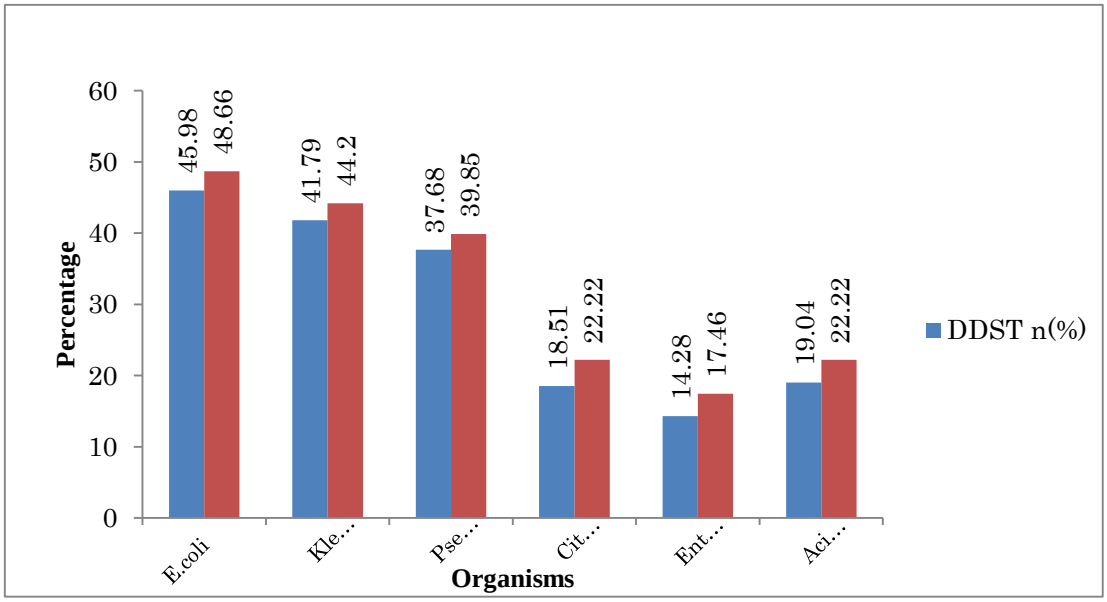

Figure 6: Comparison of DDST and PCDDT in detection of ESBL

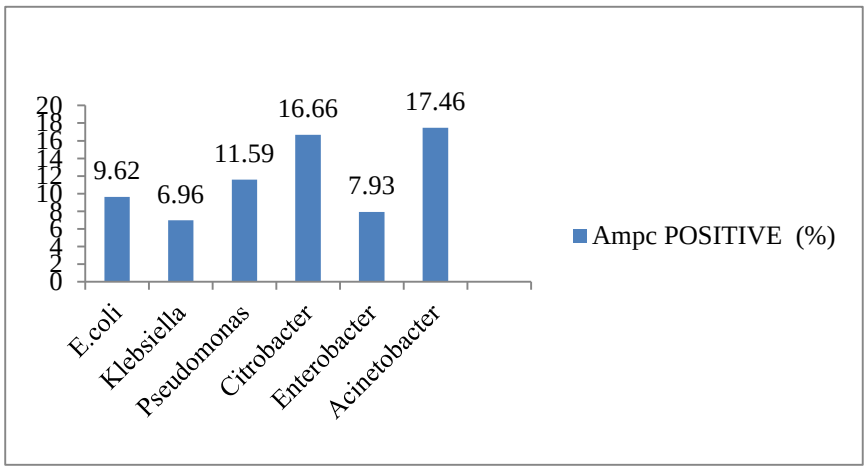

Figure 7. AmpC Positive (%)

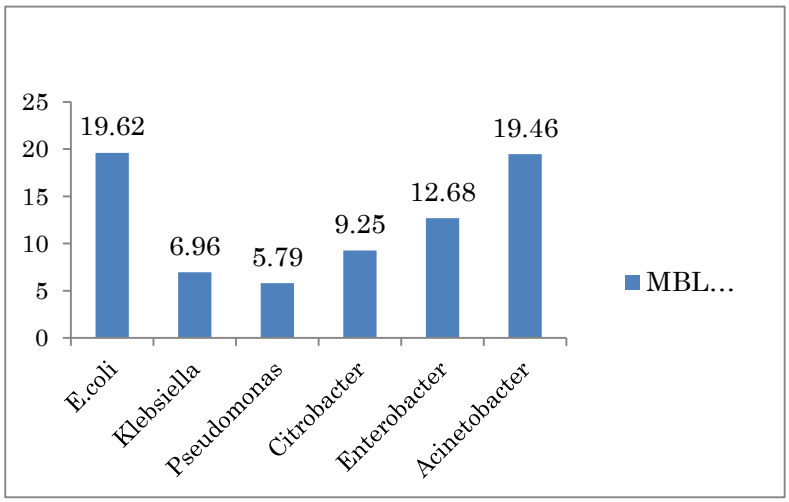

Figure 8: Distribution of MBL producer

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Table 1: Resistance pattern of β lactamase producing gram negative bacilli.

Antibiotic	E.coli n=187	Klebsiella n=201	Pseudomonas n=138	Citrobacter n=54	Enterobacter n=63	Acinetobacter n=63
Piperacillin	46(23.52)	29(14.2)	52(39.13)	31(57.40	30(47.61)	31(49.20)
Amikacin	53(22.99)	47(15.42)	37(16.66)	15(18.51)	28(17.33)	26(24.19)
Gentamicin	106(49.19)	107(42.28)	79(35.50)	28(40.74)	34(53.98)	35(55.55)
Cotrimoxazole	96(46.52)	84(20.85)	47(21.73)	19(25.92)	34(34.92)	23(19.35)
Ciprofloxacin	101(48.12)	127(51.74)	89(64.49)	20(25.2)	29(46.03)	29(46.03)
Nitrofurantoin	36(14.97)	49(41.66)	9(3.62)	10(12.96)	9(9.88)	5(4.83)
Ceftazidime	140(68.98)	118(50.74)	100(64.49)	26(42.59)	27(27.89)	22(27.41)
Cephotaxime	144(77.00)	148(73.63)	110(78.71)	34(62.92)	35(55.55)	36(57.14)
Ceftriaxone	146(78.07)	154(76.61)	112(81.15)	33(61.11)	36(57.14)	34(54.00)
Norfloxacin	72(52.17)	38(39.58)	10(50)	9 (42.85)	8(57.14)	4(36.36)
Cephoxitin	94(44.83)	61(23.38)	42(18.84)	22(25.92)	23(17.33)	26(22.58)
Imipenem	16(8.55)	17(8.45)	17(12.31)	8(13.96)	7(11.22)	12(17.74)
Amoxyclav	25(10.69)	29(8.45)	77(55.79)	6(7.47)	22(34.92)	11(17.25)
Piperacillin/Tazobactum	23(9.09)	41(20.39)	38(27.53)	8(14.81)	17(15.87)	13(11.29)

Third generation cephalosporins showed highest reistance for all gram negative isolates

Table 2: Percentage of co expression of newer β lactamases in GNB

Isolate	ESBL +Ampc	ESBL+MBL	Ampc+MBL	ESBL+Ampc+MBL
E.coli n=187	14(7.48)	8(4.27)	7(3.74)	6(3.20)
Klebsiella n=201	11(5.47)	7(3.48)	6(2.98)	6(2.98)
Pseudomonas n=138	14(10.14)	5(3.62)	12(8.69)	7(5.07)
Citrobacter n=54	8(14.81)	5(9.25)	5(9.25)	5(9.25)
Enterobacter n=63	11(17.46)	3(4.76)	4(6.34)	6(9.52)
Acinetobacter n=63	11(17.46)	6(9.52)	10(15.87)	6(9.52)
Total	69(9.77)	34(4.81)	44(6.23)	36(5.09)

Table 3: Comparison of distribution of organisms in various studies

Studies	*E.coli*	*Klebsiella spp.*	*P.aeruginosa*	*Enterobacter spp.*	*Acinetobacter spp.*	*Citrobacter spp.*
Ratna et al[49] (2003)	53.07	35.04	-	7.69	-	3.80
Franklin et al[44] (2006)	7.30	9.50	25.70	8.00	8.80	1.40
Luzzaro F et al[62] (2006)	31.9	15.10	-	7.50	-	-
Kumar MS. et al[4] (2006)	50.79	27.31	-	0.60	-	11.40
Sridhar et al[52] (2008)	35.78	13.20	-	4.40	-	-
Present study	26.48	28.47	19.54	8.92	8.92	7.64

Image 1: DDST - Synergism between inhibitory zones of 3rd generation cephalosporin disc and Amoxicillin-Clavulanic acid disc

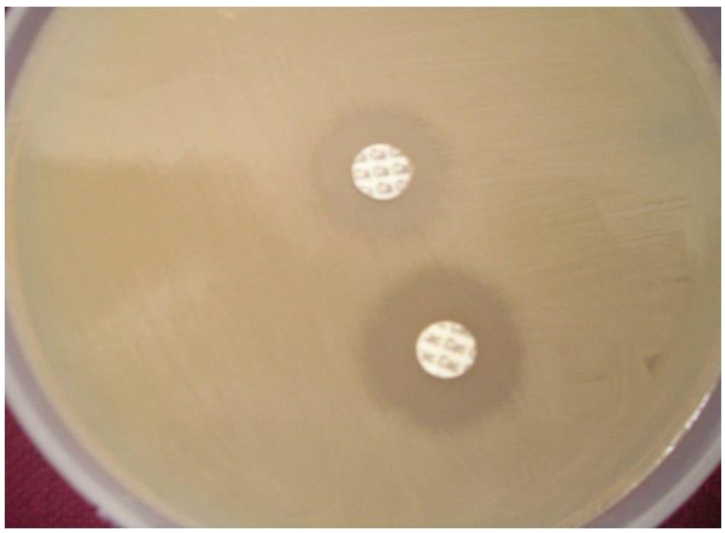
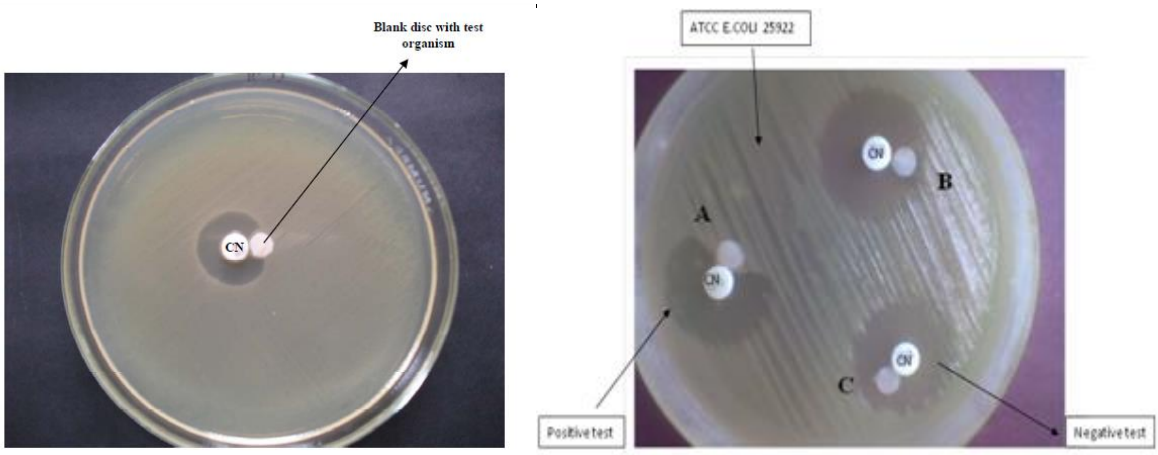


Image 2: PCDDT - >5mm increase in zone diameter for Ceftazidime- Clavulanic acid

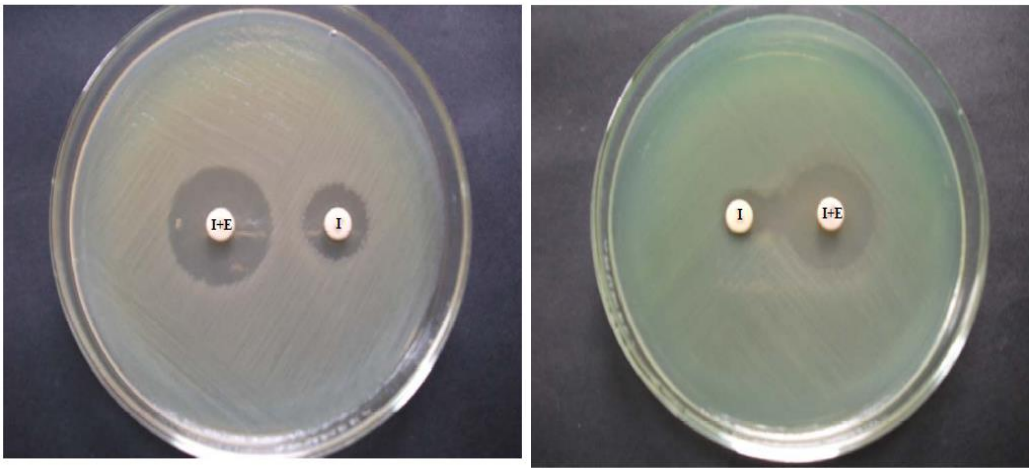
Amp C Betalactamase detection

Image-3 **Image-4**



Metallobetalactamase detection

Image 5 **Image 6**



Imipenem sensitive MBL producer. Imipenem resistant MBL producer.

Summary:

A total of seven hundred and six Gram negative clinical isolates received from Kamineni institute of medical sciences, Narketpally were screened for co-expression of ESBLs, Amp C and MBL production.

i. *Klebsiella spp* was the commonest isolate (28.47%) followed by *E.coli* (26.48%),
ii. Other isolates were *Pseudomonas aeruginosa* (19.54%), *Enterobacter spp* (8.92%), *Acinetobacter spp* (8.92%) and *Citrobacter spp* (7.64%).

iii. Gram negative isolates were tested for ESBL by DDST and PCDDT. 272 out of 706 gram negative isolates were ESBL producers. ESBL production was seen more in *E.coli* followed by *Klebsiella* spp and *pseudomonas aeruginosa*.

iv. They were tested for Amp C production by Amp C – Disc test. 73 out of 706 isolates were inducible Amp C producers. AmpC production was seen more in *Acinetobacter spp*.

v. MBL detection was done using Imipenem – EDTA combined disc test. 65 out of 706 isolates were MBL producers. MBL Production was seen more in *E.coli*

vi. Co-expression of newer β lactamases like ESBL, AmpC, MBL were found to be more in *Acinetobacter spp* and *Enterobacter spp.*

4. CONCLUSION

The incidence of infections due to organisms resistant to betalactam agents due to production of various enzymes has increased in recent years. Detection of ESBL, AmpC, and MBL production is of paramount importance both in hospital and community isolates. This is because, these strains are probably more prevalent than currently recognized. These enzymes constitute a serious threat to currently available antibiotics.Institutional outbreaks are increasing because of selective pressure due to heavy use of expanded spectrum cephalosporins and lapses in effective control measures. The coexistence of different classes of β-lactamases in a single bacterial isolate may pose diagnostic and treatment challenges. The AmpC producing organisms can act as a hidden reservoir for the ESBLs. Also, the high-level expression of the AmpC β-lactamases may mask the recognition of the ESBLs and it may result in a fatal and an inappropriate antimicrobial therapy. The high prevalence of these organisms in the ICUs emphasizes the need for an early detection of the β-lactamase producing organisms by simple screening methods, which can help in providing an appropriate antimicrobial therapy and in avoiding the development and the dissemination of these multidrug resistant strains. Judicious use of antibiotics, strict hand hygiene protocols, and implementation of appropriate infection – control measures in the hospital are necessary in preventing the spread of these multidrug resistant gram negative microorganisms. This work was done at Kamineni Institute of medical sciences, Narketpally.

5. ACKNOWLEDGEMENT

This work was done at Kamineni Institute of medicalsciences, Narketpally. I am thankful to postgraduates, faculty and technical staff who had supported in this work.

6. CONFLICT OF INTERESTT

There are no conflicts of interest

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