International Journal of Research in Medical Sciences
Kolhapure RM et al. Int J Res Med Sci. 2015 Oct;3(10):2698-2703
www.msjonline.org
pISSN 2320-6071 | eISSN 2320-6012
DOI: http://dx.doi.org/10.18203/2320-6012.ijrms20150817

Research Article

Coexpression of ESBL, Amp C and MBL in gram negative bacilli

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Received: 20 August 2015
Accepted: 03 September 2015

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ABSTRACT

Background: Resistant bacteria are emerging worldwide as a threat to the favourable outcome of common infections in community and hospital settings. Extended Spectrum Beta-Lactamases (ESBLs), AmpC β lactamases and Metallo-β Lactamases (MBL) are the three important mechanism of resistance to beta lactam drugs in the bacteria. The objective of the study was to screen gram negative isolates for co-expression of extended spectrum β-lactamase, Amp C β-lactamase and Metallo β-lactamase production.
Methods: In this study 50 (27 male & 23 female) adult skulls were investigated to determine the type of asterion, its distance from important bony landmarks and also the nearby venous sinuses were measured.
Results: Seven hundred and six isolates from various clinical samples from Kamineni institute of medical sciences Hospital, Narketpally, were processed during the period of October 2010 to September 2012. Gram negative bacilli were identified by colony morphology, gram stain, motility, enzyme detection tests, etc. ESBL detection was carried but by two procedures like double disc synergy tests (DDST) and phenotypic confirmatory disc diffusion test (PCDDT). AmpC Beta-lactamase detection was done by AmpC Disc Test. MBL production was tested by Imipenem-EDTA combined disc test.
Conclusions: *Klebsiella* was the commonest isolate (28.47%) followed by *E coli* (26.48%), *Pseudomonas aeruginosa* (19.54%), *Enterobacter* (8.92%), *Acinetobacter* (8.92%) and *Citrobacter* (7.64%). A total of 272 out of 706 gram negative isolates were ESBL producers. ESBL production was seen more in *E. coli* followed by *Klebsiella* and *P. aeruginosa*. A total of 73 out of 706 isolates were inducible Amp C producers. AmpC production was seen more in Acinetobacter. A total of 65 out of 706 isolates were MBL producers. MBL Production was seen more in *E. coli*.

Keywords: ESBL, Amp C, MBL, Resistance, Isolates, Bacteria, Superbugs, Culture and Sensitivity

INTRODUCTION

Resistant bacteria are emerging worldwide as a threat to the favourable outcome of common infections in community and hospital settings.[1] Among the wide array of antibiotics, beta-lactams are the most varied and widely used agents accounting for over 50% of all systemic antibiotics in use.

The most common cause of bacterial resistance to beta lactam antibiotics is the production of betalactamases.[1] Many of the second and third generation penicillin and cephalosporins were specifically designed to resist the hydrolytic action of major beta lactamases. However, new beta lactamases emerged against each of the new classes of beta-lactams that were introduced and caused resistance. The latest in the arsenal of these enzymes has been the evolution of Extended Spectrum Beta-Lactamases (ESBLs).[2]

These ESBLs are commonly produced by many members of Enterobacteriaceae especially *E. coli* and *Klebsiella pneumonia*.[3] Being plasmid mediated, they are easily transmitted among members of Enterobacteriaceae thus facilitating the dissemination of resistance to beta lactams. They also carry resistant genes to quinolones and aminoglycosides.

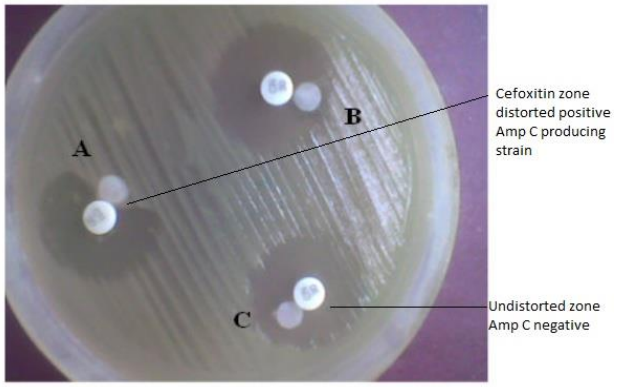

Figure 1: Amp C producer strain.

ESBLs have also been found in Pseudomonas aeruginosa and other Enterobacteriaceae strains like *Enterobacter* species, *Citrobacter* species, *Proteus* species, *Morganella morganii*, *Serratia marcescens*, *Shigelladysenteriae* and *Capnocytophagaochracea*.[4]

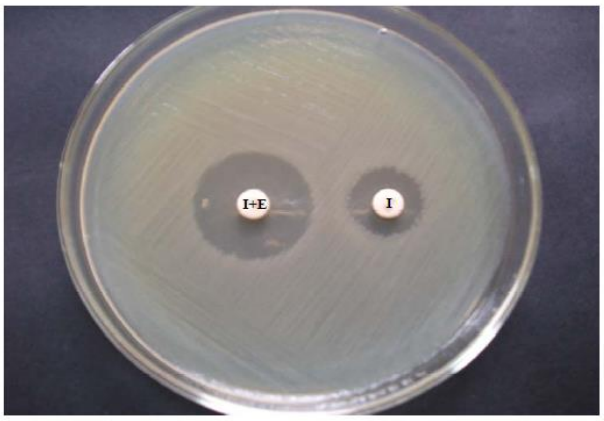

Figure 2: Metallo-Beta Lactamase detection (I= Imipenem and E= EDTA).

The chromosomally mediated beta-lactamase production is mainly through expression of Amp C gene which is either constitutive or inducible.[4] These enzymes have been described in *K. pneumoniae*, *E coli*, *Salmonella* species, *Proteus mirabilis*, *Citrobacter freundii*, *Acinetobacter* species, *Enterobacter* species and *Pseudomonas aeruginosa*.[5]

Metallo-β Lactamases (MBL) have recently emerged as one of the most worrisome resistance mechanisms owing to their capacity to hydrolyse with the exception of aztreonam, all beta lactams including Carbapenems. MBL genes first detected in *P. aeruginosa*, which in recent years, spread to members of Enterobacteriaceae.[6]

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 beta lactamases in gram negative bacilli. The following were the objectives of this study.

1. Isolation of gram negative bacilli from various clinical samples.
2. Antibiotic sensitivity testing for gram negative bacterial isolates.
3. Screening of isolates for of extended spectrum β-lactamase, Ampc β-lactamase and Metallo β-lactamase production and co expression of these enzymes in single isolate.

METHODS

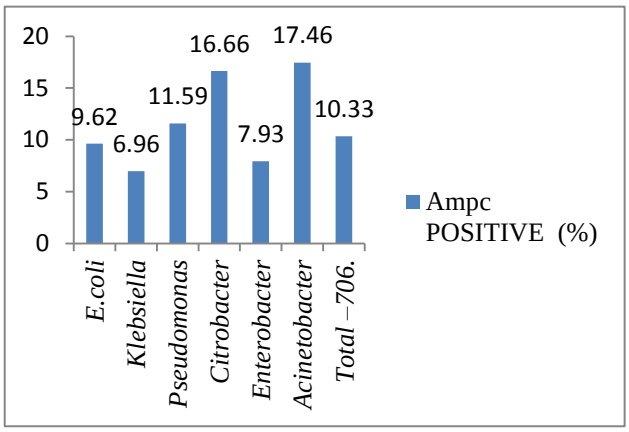

Figure 3: Amp c positivity by Ampc disc test.

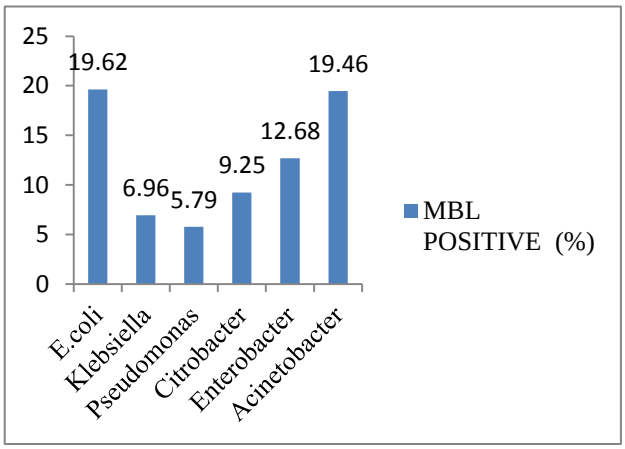

Figure 4: MBL positivity BY DPT.

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 October 2010 to September 2012. Clinical samples mainly included were urine (298), sputum (202), blood (24), pus (98), Endotracheal Tube (54) and body fluids (30).

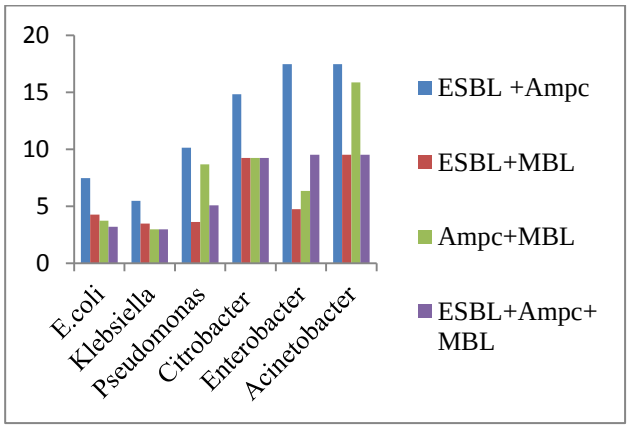

Figure 5: Percentage of co-expression of newer β lactamases in GNB.

Inclusion criteria

Only gram negative bacilli isolated from various clinical samples like blood, pus, urine, sputum, body fluids were included in the study.

Exclusion criteria

All the clinical isolates other than gram negative bacilli were excluded from the study.

Methodology

Identification of gram negative bacilli

Gram negative bacilli were identified by colony morphology, gram stain, motility, enzyme detection tests, carbohydrate metabolism based identification tests and amino acid metabolism based identification tests.

Antimicrobial susceptibility testing

The antibiotic sensitivity test was performed by modified Kirby Bauer disc diffusion technique[7, 8] with commercially available HiMedia discs according to CLSI guidelines on Mueller Hinton agar plates.

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 (20 mg amoxycillin + 10mg 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 (30 mg).

Double disc synergy test

In DDST synergy were determined between a disc of Augmentin and 30mg disc of 3rd generation Cephalosporin test antibiotic. The standardized 0.5 mc Farland 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 criteria.

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

Phenotypic confirmatory disc diffusion test

Both Cephotaxime (30mg) and Ceftazidime (30mg) disc alone and in combination with clavulanic acid (30mg) were used in this test. While performing antibiotic testing ceftazidime 30mg and ceftazidime 30mg plus clavulanic acid (30mg/ 10ml) 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. pneumonia with clavulanic acid versus its zone when tested alone was considered as ESBL producer.

Quality control

Klebsiella pneumonia ATCC 700603 (ESBL positive) was used as quality control for ESBL test.

AmpC beta-lactamase 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[5]

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 then incubated overnight at 35^0 C. Flattening of the Cefoxitin in inhibition zone in the vicinity of test disc indicates a positive test, whereas undistorted zone

indicated a negative test. The culture plates are shown in Figure 1.

Metallo-beta lactamase detection [9]

Only carbapenem resistance isolates were screened for MBL production in various studies like Behera et al, Irfan et al and Varaiya et al. In the present study irrespective of invitro susceptibility to imipenem, MBL production was tested, because correlation between invitro susceptibility to imipenem and carbapenem resistance is often imperfect.[9-11] This may be explained as either MBL genes are not always expressed or substantive resistance may require uptake of carbapenems. With the emergence of carbapenem sensitive MBL carrying organisms, the issue of which isolates to select for phenotypic MBL detection, is controversial.[9]

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 center to center 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. The culture plates are shown in Figure 2.

RESULTS

Table 1: sample wise distribution (n=706).

Sample	Number (%)
Urine	298 (42.23)
Sputum	202 (28.61)
Blood	24 (3.40)
SSI	67 (9.49)
Wound swab	15 (2.12)
Throat swab	16 (2.26)
ET tube	54 (7.62)
Body fluids	30 (4.25)

Table 2: Distribution of organisms isolated (n=706).

Organism	Number	Percentage
Esch.coli	187	26.48
Klebsiellaspp.	201	28.47
Pseudomonas aeruginosa	138	19.54
Citrobacterspp	54	7.64
Enterobacterspp	63	8.92
Acinetobacterspp.	63	8.92

The different sources of sample were listed in Table 1. Maximum number of samples were from urine (42.23%) followed by sputum (28.61%). Among these samples so many varieties of organism were isolated and the commonest isolates were *Klebsiella* (28.47%), *E. coli* (26.48%) and *P. aeruginosa* (19.54%) as shown in table 2. The resistance pattern of β lactamase producing gram negative bacilli is shown in Table 3. Comparison of DDST (Double disc synergy test) and PCDDT (Phenotypic confirmatory disc diffusion test) in detection of ESBL is shown in Table 4. ESBL detection rate was found more by PCDDT. DDST has missed 5 cases of ESBL production in E.Coli, Klebsiella. Majority of Ampc producers were Acinetobacter (17.46%), Pseudomonas (11.59 %) followed by others as shown in graph 1.Majority of MBL producers were *E. coli* (19.62%), Acinetobacter (19.46%) followed by others as shown in graph 2. Percentage of co-expression of newer all the three β-lactamases in GNB was found more in Acinetobacter (9.52%), Enterobacter (9.52%) followed by others as shown in Figure 3.

DISCUSSION

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 Amp C and 9.20% were MBL producers.

Percentage of ESBL production

In the present study 38.52% isolates were ESBL producers similar to studies by Taneja et al (36.5%) and Shukla et al (30.18%).[12,13]

Percentage of Amp C production

In our study 10.33% isolates produce inducible Amp C beta lactamases, similar to studies of Rodrigues et al (7%), less compared to the study of Sinha P et al (24%).[4,14] This shows that the chromosomally encoded Amp C beta-lactamases are prevalent in our setting.

Comparison of coexpression 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 Loveena et al (6.59%).[15]

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 (8.62%) and Loveena et al (8.79%).[16,17]

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 (3.67%).[15]

ESBL + AmpC + MBL were seen in 5.09% which is lesser than the studies done by Chatterjee et al (23.70) and Loveena et al (19.04).[15,17]

Table 3: 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
Ampicillin	46(23.52)	-	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)

β lactamase production in the present study was lower compared to other studies. This may be due to the following reasons:

1. Other studies were done in higher tertiary care centres (urban centres), whereas the present study was done in a rural medical college hospital.
2. 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.
3. Sample size and duration-other studies done were of smaller sample size and done over a short duration of time.

Table 4: Comparison of DDST and PCDDT in detection of ESBL.

ISOLATE (n)	DDST n(%)	PCDDT n (%)
E.Coli (187)	86(45.98)	91(48.66)
Klebsiella (201)	84(41.79)	89(44.2)
Pseudomonas(138)	52(37.68)	55(39.85)
Citrobacter(54)	10(18.51)	12(22.22)
Enterobacter(63)	9(14.28)	11(17.46)
Acinetobacter(63)	12(19.04)	14(22.22)

CONCLUSION

The incidence of infections due to organisms resistant to beta lactam agents due to production of various enzymes has increased in recent years. Detection of ESBL, Amp C and MBL production is of paramount importance both in hospital and community isolates. This is because,

1. These strains are probably more prevalent than currently recognized.
2. These enzymes constitute a serious threat to currently available antibiotics.
3. Institutional outbreaks are increasing because of selective pressure due to heavy use of expanded spectrum cephalosporins and lapses in effective control measures.

So, timely recognition of infection with resistant bacteria and appropriate antibiotic therapy is necessary.

Most of the isolates were resistant to beta lactam and non-beta lactam antibiotics in this study. This is because of over reliance on beta lactams and other higher antibiotics for the treatment of infections caused by gram negative organisms, empirically.

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.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Kolhapure RM, Kumar A, Rajkumar HRV. Coexpression of ESBL, Amp C and MBL in gram negative bacilli. Int J Res Med Sci 2015;3:2698-703.