RESEARCH ARTICLE

"Detection of various β-lactamases amongst gram negative bacilli from clinical isolates with special reference to Metallo β-Lactamases"

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Manuscript Info

Abstract

Introduction: Antimicrobial resistance is growing threat worldwide. Various resistance mechanisms have been found. β -Lactamase antibiotics have been the mainstay of treatment for serious infections, and most active of these are carbapenems. Predominant mechanism for resistance to β-lactam antibiotics in gram negative bacilli (GNB) is production of extended spectrum β-lactamases (ESBLs), AmpC and Metallobetalactamases (MBLs).
Aims and objectives: To detect various β-lactamases in gram negative organisms with special reference to Metallo β-lactamases (MBLs) from a tertiary care centre.
Materials and methods: Study was carried out from December 2014 to June 2015. Total number of 569 gram negative bacilli were isolated and identified according to standard guidelines. ESBL detection was done by a screening test using Ceftazidime disc which was further confirmed by a double-disc synergy test using Piperacillin-Tazobactum/ Cefipime. AmpC were screened by a cefoxitin disc, confirmed by Cefoxitin-cefotaxime disk antagonism. Carbapenemase detection was done with Imipenem screening test which were further subjected to MBL production testing.
Result: Amongst 569 isolates, 177(31.1%) were ESBL producers, 51 (9%) were AmpC producers, 31(5.5%) were ESBL and AmpC co-producers and 116(20.4%) were carbapenemase producers. Out of total 116 carbapenemase producing strains 110 (94.8%) were positive for carbapenemase production by Modified Hodge Test. In phenotypic methods for MBL production, Meropenem-EDTA disc method detected 85 (73.3%) isolates followed by Imipenem-EDTA 68 (58.6 %) and Ceftazidime-EDTA 52 (44.8%).
Conclusion: It is need of the hour to standardise various cost effective and time saving methods to detect these resistance mechanisms with routine AST in Clinical Microbiology laboratory.

Introduction:-

Antimicrobial resistance is a growing threat worldwide. Resistance mechanisms have been found for every class of antibiotic agents. Accurate and timely detection of these resistant mechanisms is very important in deciding the appropriate treatment schedule. Detection of the resistant mechanisms is always a serious challenge to the clinical laboratories (Doddaiah *et al.,* 2014).

β -Lactamase antibiotics have been the mainstay of treatment for serious infections, and the most active of these are the carbapenems (Deshmukh *et al.,* 2011). The predominant mechanism for resistance to the β-lactam antibiotics in gram negative bacilli (GNB) is the production of extended spectrum β-lactamases (ESBL). ESBL's are mutant, plasmid mediated β-lactamases derived from older broad-spectrum β-lactamases which have an extended profile that

permits hydrolysis of all cephalosporins, penicillins and aztreonam. AmpC class β-lactamases are cephalosporinase that are poorly inhibited by clavulanic acid. They are differentiated from other ESBLs by their ability to hydrolyze cephamycins as well as other extended spectrum cephalosporins (Hyder *et al.,* 2014). Metallo β-lactamases (MBLs) producing bacteria can hydrolyze a wide range of β-lactam antibiotics including penicillins, cephalosporins, carbapenems, cephamycins, but lack the ability to hydrolyze aztreonam. Moreover, their catalytic activities are generally not neutralized by commercially available β-lactamase inhibitors such as clavulanate, tazobactam, and sulbactam.

Laboratory detection of resistance to carbapenems has been reported to be difficult for many reasons; low expression of such resistance, degradation of the drug, the use of automated methods for identification and susceptibility testing, in addition to the lack of standardized methods of detection (Babey *et al.,* 2009). The present study was carried out to detect the various β-lactamases in gram negative organisms with special reference to Metallo β-lactamases (MBLs) from a tertiary care centre.

Materials and Methods:-
The study was conducted over a period of six months from December 2014 to June 2015 at a tertiary care centre. A total of 569 gram negative bacteria (GNB) were isolated from various clinical specimens and identified according to standard guidelines (Konemann *et al.,* 2006, Mackey *et al.,* 2006). Antibiotic susceptibility testing (AST) was performed on Mueller–Hinton agar plates with commercially available discs (Hi media, Mumbai) by Modified Kirby-Bauer (Bauer *et al.,* 1966) disc diffusion method and interpreted as per the CLSI 2015 guidelines. The following antibiotics were tested: Ampicillin (10 μg), Cefazolin (30 μg), Cephalothin (30 μg),Cefuroxime (30 μg) Cefotaxime (30 μg), Ceftazidime (30 μg), Cefepime (30 μg), Amoxycillin-clavulanate (20/10 μg), Piperacillin-tazobactum (100/10 μg), Gentamicin(10 μg), Amikacin (30 μg), Ciprofloxacin (5 μg), Levofloxacin (5 μg), Doxycycline (30 μg), Imipenem (10 μg), Meropenem (10 μg), Colistin (10 μg), Polimixin B(300 units). The detection methods of betalactamases were applied on the same day along with routine AST.
 Screening and Confirmatory test for ESBL detection:-

Initial screening test for ESBL production was performed on Muller Hinton agar (MHA) by disk diffusion method using ceftazidime (30 μg) disk. The lawn culture of 0.5 McFarland inoculum of the test strain was exposed to a disk of ceftazidime. After incubation at 37°C for 16-18 hours, the zone diameter ≤ 22 mm indicated ESBL production

They were further confirmed by a double-disc synergy method. Muller Hinton agar plates were swabbed to form a lawn culture with 0.5 McFarland standard inoculum of the test strain. On the MHA plate, a disk of cefotaxime (30 μg) was placed 20 mm apart, centre to centre, from amoxyclav (20/10 μg) disk whereas piperacillin-tazobactam (100/10 μg) disk was placed 20 mm apart, centre to centre from cefepime (30 μg) disk. Plates were incubated at 37°C overnight and were examined for enhancement of zone inhibition of cefotaxime and cefepime at the side facing amoxyclav and piperacillin-tazobactam disk respectively [Figure 1]. Organisms that showed a clear extension of inhibition zone towards the disk of amoxyclav and piperacillin-tazobactam were considered ESBL positive (CLSI 2015, Khan *et al.*, 2008)

Screening and Confirmatory test for Amp C detection:-
Screening for Amp C production was done by using cefoxitin disk (30 μg) on MHA. The lawn culture of 0.5 McFarland inoculum of the test strain was exposed to a disk of cefoxitin. After overnight incubation at 37°C, the zone diameter ≤ 18 mm were suspected as AmpC producer (Jacoby *et al.,* 2009). Further they were confirmed by Cefoxitin-cefotaxime disk antagonism for inducible AmpC detection. A lawn culture of 0.5 McFarland inoculum of the test strain was exposed to a disk of cefotaxime (30 μg) and cefoxitin (30 μg) placed at a distance of 1.5 cm from edge to edge [Figure 2]. After overnight incubation, there was flattening of radius of zone of inhibition produced by cefotaxime on the side nearest the cefoxitin disk in case of AmpC β-lactamase (Miles *et al.*, 2006).

Screening and Confirmatory test for Carbapenemase detection:-
Carbapenemase production was tested by performing both initial screening test and phenotypic confirmatory test. In initial screening test, lawn culture of 0.5 McFarland inoculum of the test strain was exposed to a disk of imipenem (10 μg). *E. coli* ATCC 25922 was used for quality control. After overnight incubation, the zone diameter around 16-21 mm indicated carbapenemase production (CLSI 2015). All these isolates were subjected to modified Hodge test (MHT) for confirmation of carbapenemase production. A 0.5 McFarland standard suspension of *E. coli* ATCC 25922 was prepared in a saline and it was further diluted in 1:10 in saline. The MHA plate was inoculated as per the

routine disk diffusion procedure. The plate was allowed to dry 3 to 10 minutes. A single disk of meropenem (10 µg) was placed on the plate at the centre. Using a 10-µl loop, 3–5 colonies of test organism grown overnight on a blood agar plate were picked up. They were inoculated in a straight line (at least 20–25 mm in length) out from the edge of the disk [Figure 3]. The plate was incubated at 37^0C for 16-20 hours. Following incubation, the MHA plate was examined for enhanced growth around the test organism streak at the intersection of the streak and the zone of inhibition. The enhanced growth suggests positive test for Carbapenemase production (CLSI 2015). The carbapenemase producing strains were further subjected to three different phenotypic methods for detection of MBL production.

MBL production- Combined disk test (Disk potentiation test-DPT):-
It was used to detect metallo-β-lactamase (MBL) production (Picao *et al.*, 2008). In this test, the lawn culture of 0.5 McFarland inoculum of the test strain was exposed to a disk of imipenem (10 µg) and imipenem-EDTA (10/750 µg). The difference of ≥ 7 mm in zones of inhibitions of two disks indicated MBL production. Since EDTA may have some bactericidal activity, a blank disc of EDTA was tested as control [Figure 4]. DPT was performed with meropenem/ meropenem- EDTA and ceftazidime/ceftazidime- EDTA also (Pitout *et al.*2005).

Result:-
Total number of gram negative bacilli studied was 569. Out of these 569, 344 (60.5%) showed the presence of one or combination of enzymes and 225(39.5%) were negative for betalactamases. Amongst 569 isolates, 177(31.1%) were ESBL producers, 51 (9%) were AmpC producers, 31(5.5%) were ESBL and AmpC co-producers and 116(20.4%) were carbapenemase producers [Table 1]. Out of 177 ESBL producers, maximum were *E.coli* (46.9%) followed by *Klebsiella* species (43.1%) *Nonfermenter* spp. (36%) and *Pseudomonas* (27%). The phenotypic detection of ESBL production was attempted by beta-lactam and beta-lactamase inhibitor combination like amoxyclav and cefotaxime and piperacillin/ tazobactam and cefepime.

AmpC production was highest among *Nonfermenter* (16%) followed by *Klebsiella* species (15.3 %) and *E.coli* (14.2%). Out of 51 AmpC producer, 17(33.3%) were inducible and 34(66.6%) were stably derepressed mutants.

Carbapenemase production was seen in 116 (20.4%) of the isolates. Highest number was seen amongst *Acinetobacter* species (36.4%) followed by *Klebsiella* species (34.7%), *Pseudomonas* (30.2%) and *Nonfermenter* (20%). They were further confirmed by MHT. Out of total 116 carbapenemase producing strains 110 (94.8%) were positive for carbapenemase production in MHT. In the phenotypic methods for MBL production [Table 2], Meropenem-EDTA disc method detected 73.3% isolates followed by Imipenem-EDTA (58.6 %) and Ceftazidime-EDTA (44.8%).

Discussion:-
The newer β-lactamases like ESBL, AmpC and Carbapenemase has emerged as a cause of antibiotic resistance among the GNB worldwide in the recent years. Failure to detect these enzymes has contributed to their uncontrolled spread and sometimes to therapeutic failure (Doddaiah *et al.*2014). Study by Nagdeo *et al.*(Nagdeo *et al.,*2012) found ESBL production in 39.03 strains, AmpC in 9.29%, and MBL in 7.44%strains which is highly comparable to our study.

In the present study, ESBLs were found in 31.1% isolates which is comparable with Doddaiah *et al.* (Doddaiah *et al.,* 2014) and Singh *et al.* (Singh *et al.,* 2012) as shown in table 3. Maximum number of ESBL production was found among *E.coli* (46.9%) and *Klebsiella spp.* (46.1%) followed by *Nonfermenters* (36%) and *Pseudomonas* (27%). Similar findings are reported by other studies (Doddaiah *et al.,* 2014, Haider *et al.,* 2014). This high incidence of ESBLs in *E. coli* may be peculiar to the Indian subcontinent. Although CLSI recommendations exist, but they are limited to *E. coli* and *Klebsiella spp.* (CLSI 2015). Haider *et al.* (Haider *et al.* 2014)*,* found pseudomonas to be the second commonest ESBL producer after *E.coli*. In our study also, *Pseudomonas* and *Nonfermenters* were found to be the remarkable ESBL producers. These figures reinforce the fact that ESBL production should be routinely screened in Gram-negative bacteria other than *E. coli* and *Klebsiella.* Piperacillin/tazobactam with cefepime was found to be more sensitive as compared to amoxyclav/clavulanic acid with cefotaxime. This finding is comparable with study done by Khan MKR, Thukral SS (Khan *et al.,* 2008) who reported cefepime / piperacillin/tazobactam to be the most sensitive test for ESBL detection in AmpC-positive *P. mirabilis*. Hyder *et al.* (Hyder *et al.,* 2014) reported similar findings.

In our study, AmpC were found in 9% isolates which is comparable to Singhal *et al.,* (8%) (Singhal *et al.*, 2005) and Valsan C *et al.* (10%) (Valsan C *et al.*, 2013). It is slightly lesser as compared to a study done in 2011 by Shoorshetty *et al.* (33.55 %) (Shoorshetty *et al.* 2011). But the authors used boronic acid disk test in combination with the CLSI phenotypic confirmatory test for the identification of ESBL and AmpC among *Enterobacteriaceae* which is highly sensitive and specific.

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 (Oberoi *et al.,* 2013). We detected coproduction of ESBL and AmpC in 31 isolates (5.5%) which is similar to Raffie *et al.,* (3.9%) (Raffie *et al.,* 2014) but lower than other reports (24.5-26%) (Shreeshma *et al.,* 2013). Detection of ESBLs in AmpC producing Gram-negative bacteria is often a problem. High level expression of AmpC can prevent recognition of ESBLs leading to false negative results (Raffie *et al.,* 2014).

In the present study 20.4 % of isolates were carbapenemase positive which is compared with studies done by Doddaiah *et al.,* (18.25%) (Doddaiah *et al.,* 2014), Babey *et al.,* (34.2%) (Babey *et al.,* 2009) as shown in table 3. Until the last few years imipenem and meropenem has been the most reliable agent for treating serious infections caused by MDR nosocomial bacteria such as *Acinetobacter baumannii* and *P. aeruginosa*. However, recent reports have documented the worldwide emergence of clinical isolates of *Acinetobacter spp.*, *P. aeruginosa* and other members of the *Enterobacteriacae* with acquired carbapenemases. This has an important therapeutic and infection control implications as these strains are difficult to treat by β-lactamase inhibitors and resistance can spread widely into various Gram negative bacilli (Babey *et al.,* 2009).

MHT identified 94.8% of strains as carbapenemase producer in our study. Bartolini *et al.,* (Bartolini *et al.,* 2014) found MHT to be 94% sensitive and 100% specific for identification of carbapenemase production. As per CLSI 2015 , MHT have some limitations like false positives in isolates producing ESBL or AmpC enzymes coupled with porin loss and false negative in NDM producing isolates. But MHT is a phenotypic test used to detect carbapenemase in isolates demonstrating elevated but susceptible carbapenem MICs and has demonstrated sensitivity and specificity exceeding 90% in identifying carbapenemase producing *Enterobacteriaceae* (CLSI 2015).

In present study, carbapenemase production was highest in *Acinetobacter spp.* (36.4%) followed by *Klebsiella spp.* (34.7%) and *Pseudomomonas spp.*(30.2%). Deshmukh *et al.* (Deshmukh *et al.* 2011) detected *Pseudomonas* to be the commonest carbapenemase producer (36.8%) followed by *Klebsiella pneumonia* (31.6%) and *A.baumannii* (21%). Pandya *et al.* (Pandya *et al.* 2011) detected highest carbapenemase production in *Pseudomomonas* (9.92%), followed by *Klebsiella* (7.26%) and *Acinetobacter spp.* (7.14%). Bora *et al.* (Bora *et al.* 2014) detected carbapenemase production in 18.98% isolates of *E.coli* and 21.08% of *K.pneumoniae*. But in this study only *enterobacteriaceae* were included. Our study is comparable to Nagdeo *et al.,* (Nagdeo *et al.*2012) who showed *Nonfermenters* to be the commonest followed by *Pseudomonas* and *Enterobacteriaceae* for carbapenemase production. Noyal *et al.,* (Noyal *et al.,* 2009) found carbapenemase production in 53% isolates of *Acinetobacter spp.* which is much higher than ours but 27.8% isolates of *Pseudomonas spp.* which is comparable.

The carbapenemase producing isolates were further subjected to three phenotypic methods for MBL detection. Over the past few years, MBL producing gram negative bacteria are being reported with increasing frequency from several parts of the world and have emerged as a most widespread and clinically significant carbapenem resistance mechanisms (Bora *et al.,* 2014). The detection of MBL-producing isolates by PCR is expensive, requires specialized technicians and instruments, and, more importantly, is able to detect only previously described MBL-encoding genes. However, the detection of the MBL phenotype of resistance is of crucial importance for selecting the most appropriate therapy and applying infection control measures. For these reasons, an accurate and easy-to-perform phenotypic test is desirable and urgently necessary in hospitals with a high prevalence of MBL-producing isolates (Picao *et al.,* 2008).

In our study, the rate of MBL production in gram negative bacilli was 14.7% (85/579). MBL production has been reported from various regions ranging from 7-65% (Deshmukh *et al.,* 2011). Most of these studies reported MBL production in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Very few studies have reported MBL production in *Enterobacteriaceae*. In our study, we reported 44 strains of MBL producing *Enterobacteriacae*. Of the 116 carbapenem -resistant strains, thirty one strains did not show MBL production. The likely reasons for

carbapenem resistance in non MBL producers varied such as decreased cell membrane permeability, or activity of efflux pumps (Tellis *et al.,* 2013).

In our study MEM (73.3%) and IMP (58.6%) were better than CAZ (44.8%) when combined with EDTA in DPT for detection of MBL. Picao *et al.,* (Picao *et al.,* 2008) reported similar findings. Kumar *et al.,* (Kumar *et al.,* 2012) and Manoharan *et al.,* (Manoharan *et al.,* 2010) found I/I-EDTA to be the better method for routine MBL detection than MEM/MEM-EDTA. Few other studies also reported I/I-EDTA as most sensitive method for detection of MBL production in gram negative bacilli (Pandya *et al.,* 2011). Noyal *et al.,* (Noyal *et al.,* 2009) and a study from National Institute of Infectious Diseases, Tokyo, Japan (Arakawa *et al.,* 2000) found CAZ/EDTA-CAZ combination to be better than MEM/MEM-EDTA combination. Our results were in contrary with the above mentioned studies. Our study is highly comparable with Sheikh *et al.* (Sheih *et al.,* 2014) and Buchunde *et al.* (Buchunde *et al.,* 2012). Both these studies (Sheih *et al.,* 2014, Buchunde *et al.,* 2012) have compared their results of DPT with the molecular methods. Overall, a combined disk diffusion testing with MEM and MEM-EDTA is easier, cost-effective and sensitive, and can be easily introduced on the same plate of Antibiotic susceptibility testing done routinely in the clinical laboratory.

Our study was limited by the fact that no molecular confirmation was done for above mentioned resistance mechanisms due to constraint of resources. But in a resource-poor developing country, these innovative disc placement methods are easy, affordable and reliable for detection of various beta-lactamases. Our study showed betalactamase production in a considerable number of gram negative bacilli. CLSI guidelines explain the method of ESBL detection in Enterobacteriaceae. But in our study we got variable number of ESBL producing Pseudomonas and Acinetobacter. Similarly guidelines for MBL detection are limited. Hence our study emphasises the importance of ESBL detection in isolates other than Enterobacteriaceae and MBL detection with CDT in gram negative bacilli.
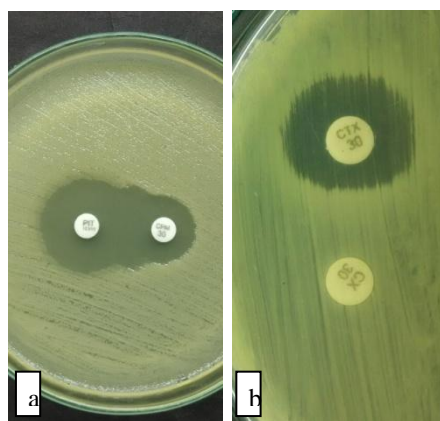

Fig.1a: Double disk synergy tests for ESBL detection: Fig. shows enhancement of zone of inhibition of cefepime towards piperacillin-tazobactam disk. b: Cefoxitin-cefotaxime disk antagonism test for AmpC detection: Fig. shows flattening of zone of inhibition produced by cefotaxime adjacent to cefoxitin disk.
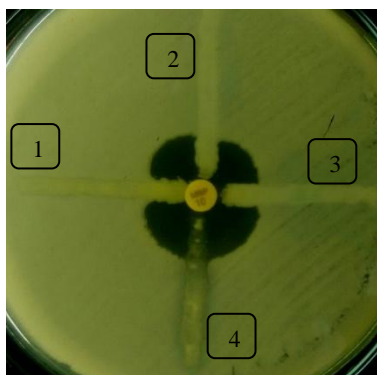
Fig 2: Modified Hodge Test: Fig. shows enhanced growth at the intersection of zone of inhibition and test organism steak 1,2 & 3; while no. 4 is negative for carbapenemase production.
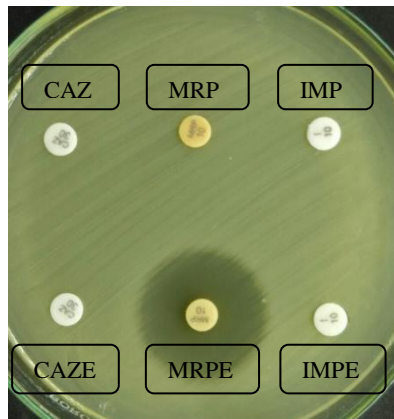

Fig 3: Combined Disc Test: CAZ: Ceftazidime, CAZE: Ceftazidime-EDTA , MRP: Meropenem, MRPE:Meropenem-EDTA, IMP: Imipenem, IMPE: Imipenem-EDTA

Table 1: Distribution of ESBL, AmpC, and Carbapenemase producers among the isolates

Isolate	ESBL (%)	AmpC (%)	ESBL+AmpC(%)	Carbapenemase (%)
E.coli (226)	106(46.9)	32(14.2)	23(10.2)	11(4.9)
Klebsiella species(72)	31(43.1)	11(15.3)	7(9.7)	25(34.7)
Citrobacter species(35)	8(22.9)	4(11.4)	1(2.9)	7(20)
Proteus(9)	1(11.1)	0(00)	0	1(11.1)
Enterobacter species (5)	0(00)	0(00)	0	0(00)
S.Typhi (2)	0(00)	0(00)	0	0(00)
Nonfermenters(25)	9(36)	4(16)	0	5(20)
Pseudomonas(63)	17(27)	0 (00)	0	19(30.2)
Acinetobacter Species(132)	5(3.8)	0(00)	0	48(36.4)
Total (569)	177(31.1)	51(9)	31(5.5)	116(20.4)

Table 2: Metallo-beta-lactamases (MBL) by Combined disk test (n=116)

Combined disk test	MBL production
Meropenem / Meropenem EDTA	85(73.3 %)
Imipenem/ Imipenem EDTA	68 (58.6 %)
Ceftazidime/ Ceftazidime EDTA	52 (44.8 %)

Table No. 3: ESBLs, AmpC and Carbapenemase in GNB as reported by various workers

Authors	ESBL	AmpC	Carbapenemases
Ahmed S 2014	61.6%	6.8%	34.2%
Doddaiah 2014	33.86%	14.24	18.25
Yusuf 2014	14.4%	11.9%	10.3%
Valsan C 2013	60	10	12.6
Singh R K 2012	27.2	17.7	-
Shoorshetty 2011	68.86%	33.5	-
Singhal 2005	64%	8%	-

Conclusion:-

Carbapenems are considered the last line of effective therapy available for the treatment of severe infections. Potentially toxic and costly drugs like tigecycline, colistin and polymixin B are the only limited options against MDR or resistant MBL-producing isolates. Failure to identify them may lead to inappropriate therapy, treatment

failure and may result in increased mortality. Thus it is the need of the hour to standardise various cost effective and time saving methods to detect these resistance mechanisms with routine AST in a Clinical Microbiology laboratory.

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