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A STUDY ON PREVALENCE OF METALLOBETALACTAMASE PRODUCING GRAM NEGATIVE BACILLI IN A TERTIARY CARE HOSPITAL

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Abstract : AIM- To estimate the prevalence of MetalloBeta Lactamase (MBL) producing gram negative bacilli (GNB) isolated from hospitalized patients and to determine the Minimum Inhibitory Concentration (MIC) of these isolates to imipenem. MATERIALS AND METHODS- A total of 3204 GNB were isolated from various specimens over a period of 6 months. These isolates were subjected to antimicrobial susceptibility testing by disc diffusion test as per Clinical and Laboratory Standards Institute(CLSI) guidelines. A total of 1587 isolates which were resistant to ceftazidime were taken up for further study. Modified Hodge Test (MHT) was performed to screen for MBL production by the isolates followed by testing with Combination Disc Test (CDT). Isolates exhibiting an increase in zone size in CDT were identified as MBL producers. MIC of these MBL producing isolates to imipenem was determined by macrobroth dilution method as per CLSI guidelines. Appropriate control strains were used while performing all the tests. RESULTS- Of the 1587 ceftazidime resistant isolates studied, 1301 were members of Enterobacteriaceae, 188 were Pseudomonas aeruginosa and 98 were Acinetobacter baumanii. MHT was positive in 78, 26 and 14 of these isolates respectively. However, only 60 of the 78 Enterobacteriaceae, 12 of the 14 A.baumanii and all 26 P.aeruginosa were identified as MBL producers by CDT. Majority of the MBL producing bacilli were isolated from tracheal aspirates. Escherichia coli was the predominant MBL producing member of Enterobacteriaceae. Significant statistical association was observed between isolates being non-fermenting bacilli and the production of MBL (p value less than 0.0001). Most of the Enterobacteriaceae isolates exhibited MIC of 16-32 mcg per ml to imipenem while most P.aeruginosa and A.baumanii isolates had MIC of 32-64 mcg per ml. The overall prevalence of MBL production in gram negative bacilli was 3.1 percent with the highest prevalence of 8.5 percent being observed in P.aeruginosa followed by 6.4 percent in A.baumanii and 2.2 percent in Enterobacteriaceae. CONCLUSION- The observation of MBL production by various gram negative bacilli emphasizes the need for regular screening of ceftazidime resistant isolates for possible production of MBL. This would help in guiding patient therapy and institute appropriate infection control measures.

Keyword : Enterobacteriaceae, P.aeruginosa, A.baumanii, MBL, Modified Hodge Test, Combination Disc Test

INTRODUCTION : There has been an alarming rise in the resistance pattern of gram negative bacilli (GNB) to antimicrobial agents in the past few decades. The spread of carbapenemases particularly Metallo--Lactamases (MBLs) is posing serious therapeutic challenges to the medical fraternity. GNB commonly implicated in producing MBLs include *Pseudomonas* spp, *Acinetobacter* spp and members of Enterobacteriaceae family.[1]MBLs belong to Ambler class B enzymes and possess a broad hydrolytic profile that includes all lactam agents/ lactam inhibitors with the exception of monobactams. MBL enzymes require divalent cations as cofactors for their activity and are inhibited by the action of a metal ion chelator like Ethylene Diamine Tetra Acetic acid (EDTA), Mercapto Propionic Acid (MPA) and Sodium Mercapto Acetic acid (SMA).[2]The further treatment options of MBL producing bacilli are limited to very few drugs like Tigecycline, Polymyxin B or Colistin. Another prime concern is that these MBL genes are located on mobile genetic elements (plasmids/ transposons) enabling their easy dissemination among gram negative bacillary species.The first MBL gene bla_{IMP-1} was first discovered in *Serratia marcescens* in 1991 in Japan followed by identification of bla_{VIM-1} and bla_{KPC} in Greece and United States respectively.[2] Till now 24 different types of MBL have been described globally involving various organisms and their numbers are continually increasing. With the worldwide increase in the occurrence, types, and rate of dissemination of MBLs, early detection is critical. Accurate detection of MBL producing organisms is of paramount importance to initiate appropriate treatment of the patients and also implement infection control measures in the hospitals. This study was undertaken to identify and estimate the prevalence of MBL producing GNB using simple and sensitive methods.

MATERIALS AND METHODS

The study was carried out over a period of 6 months (January-June 2012) at a tertiary care hospital after obtaining prior clearance from the Institutional Ethics Committee. Various GNB's were isolated from clinical specimens like pus, urine, sputum, blood, tracheal aspirates, body fluids and bronchial washings. The bacterial isolates obtained in culture were identified by their morphology on Nutrient agar, Blood agar, MacConkey agar; Gram's stain characteristics, motility, biochemical tests like production of catalase, indole, urease, reaction to oxidase reagent, utilization of citrate and fermentation of Triple Sugar Iron medium.

In this study, the following algorithm was used for detection of MBL production by bacterial isolates

Resistance to ceftazidime in isolates with a negative MHT may be due to production of ESBL, AmpC or other mechanisms. Isolates with positive MHT but negative CDT are probable producers of carbapenemases other than MBL.

ANTIMICROBIAL SUSCEPTIBILITY TESTING: Antimicrobial susceptibility testing was done on Mueller-Hinton agar (MHA) [Hi-media laboratories, Mumbai, India]. Four to five colonies of pure growth from an overnight culture of organism was transferred to a tube containing 4-5 ml of peptone water and incubated at 37ºC for 2 hrs and adjusted to match 0.5 McFarland turbidity and a lawn culture of the isolate was made with sterile cotton swab. Antibiotic discs (Hi-Media Laboratories, Mumbai, India) were placed at appropriate distance on the lawn culture and incubated at 35 ± 2ºC in ambient air for 16-18 hours.[4] Standard strains of *E.coli* (ATCC 25922) and *P.aeruginosa* (ATCC 27853) were used as controls and tested in a similar way. The zone of inhibition was measured carefully and interpreted according to Clinical Laboratory Standard Institute (CLSI) 2011 guidelines.[5] The antibiotics tested were amikacin (AK-30µg), gentamicin (G-10µg), ciprofloxacin(CIP-30µg), ceftazidime (CZ-30µg), piperacillin-tazobactam (PT-100+10µg), and imipenem (IPM-10µg).

MODIFIED HODGE TEST (MHT): A 0.5 McFarland of ATCC *E.coli* 25922 was prepared by direct colony suspension method, it was diluted 1:10 in normal saline, and lawn culture was made. Plate was allowed to dry for 5 to 10 minutes, and imipenem disc was placed in the centre of the plate. 3-5 colonies of test and QC organism (ATCC *Pseudomonas aeruginosa* 27853) grown overnight on a blood agar plate were taken with a 4 mm loop and inoculated in a straight line of 20-25 mm out from the edge of the disc. The plate was incubated for 16- 20 hours at 35 ± 2 ºC in ambient air. Enhanced growth at the intersection of streak and zone of inhibition was interpreted as positive for possible MBL production. As carbapenemase produced by the test organism inactivated the imipenem which diffused into the medium, growth of ATCC *E.coli* 25922 was enhanced around the MBL producing test organism.[5,6,7]

COMBINATION DISC TEST(CDT): Discs of imipenem (IPM-10µg) and imipenem-EDTA (IE-10+750µg) were placed on a MHA plate inoculated with 0.5 McFarland turbidity of the test isolate and incubated at 35 ± 2 ºC for18- 24 hours. Any isolate showing an increase in zone size of at least 7 mm around the imipenem-EDTA disc compared to imipenem disc alone was recorded as a MBL producing strain.[8,9]*E.coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) were used as control strains.[10]

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) OF IMIPENEM FOR MBL PRODUCING ISOLATES: All isolates which were found to be MBL producers were tested by Macrobroth dilution method for their MIC against imipenem.Standard strains of *E.coli* (ATCC 25922) and *P.aeruginosa* (ATCC 27853) were used as controls. Results were interpreted as per CLSI 2011 guidelines.[5]For Enterobacteriaceae, MIC values of ≤1µg/ml, 2µg/ml and ≥4µg/ml were interpreted as sensitive, intermediate and resistant to imipenem respectively while for Non fermenting GNB, the corresponding values were ≤4µg/ml, 8µg/ml and ≥16µg/ml respectively.

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) version 20.0 . The proportional data of this study were tested using Pearson's Chi square analysis test [2]

RESULTS

CHART 1- SUSCEPTIBILITY PATTERN OF CEFTAZIDIME (CZ) AMONG THE ISOLATED GRAM NEGATIVE BACILLI (n=3204)

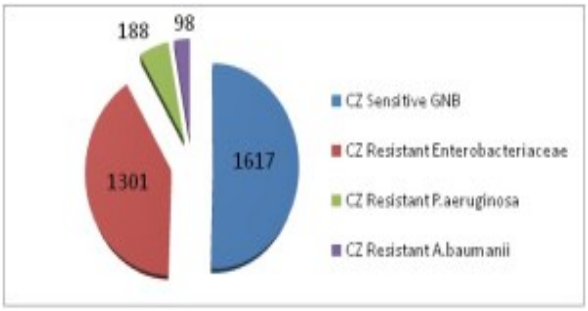

CHART 2- RESULTS OF MHT AND CDT IN CEFTAZIDIME RESISTANT ISOLATES OF ENTEROBACTERIACEAE (n=1301)

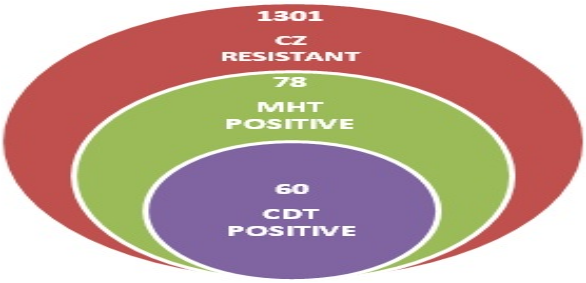

CHART 3- DISTRIBUTION OF MBL PRODUCING STRAINS AMONG ENTEROBACTERIACEAE (n= 60)

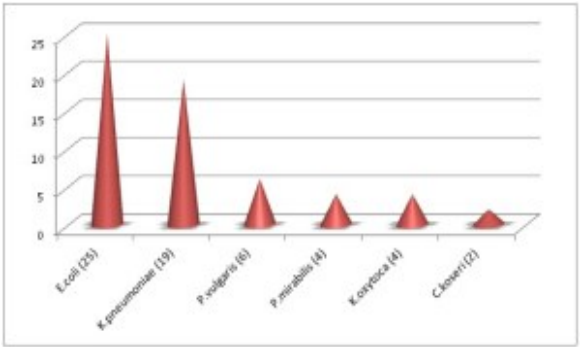

CHART 4- RESULTS OF MHT AND CDT IN CEFTAZIDIME RESISTANT ISOLATES OF PSEUDOMONAS AERUGINOSA (n=188)

CHART 5- RESULTS OF MHT AND CDT IN CEFTAZIDIME RESISTANT ISOLATES OFACINETOBACTER BAUMANII (n=98)

CHART 6- DISTRIBUTION OF MBL PRODUCING ISOLATES WITH RESPECT TO CLINICAL SAMPLES (n=98)

TABLE 1- DISTRIBUTION OF MBL PRODUCERS AMONG THE ISOLATED GRAM NEGATIVE BACILLI (n= 3204)

GNB (n)	MBL PRODUCING (n)	NON- MBL PRODUCING (n)
Enterobacteriaceae (2712)	60	2652
P.aeruginosa(305)	26	279
A.baumanii (187)	12	175
	98	**3106**

TABLE 2- MIC VALUES OF MBL PRODUCING ENTEROBACTERIACEAE TO IMIPENEM (n=60)

GNB	MIC (µg/ml)							
	1	2	4	8	16	32	64	128
E.coli (n=25)	-	-	2 (8%)	4 (16%)	8 (32%)	6 (20%)	3 (12%)	2 (8%)
K.pneumoniae (n=19)	-	-	1 (5.3%)	2 (10.5%)	7 (36.8%)	6 (31.6%)	2 (10.5%)	1 (5.3%)
P.vulgaris (n=6)	-	-	-	1 (16.7%)	2 (33.3%)	2 (33.3%)	1 (16.7%)	-
P.mirabilis (n=4)	-	-	-	-	1 (25%)	2 (50%)	1 (25%)	-
K.oxytoca (n=4)	-	-	-	1 (25%)	2 (50%)	1 (25%)	-	-
C.koseri (n=2)	-	-	-	-	-	2 (100%)	-	-

TABLE 3- MIC VALUES OF MBL PRODUCING P. AERUGINOSA TO IMIPENEM (n=26)

	MIC (µg/ml)							
	4	8	16	32	64	128	256	512
No. of Isolates	-	-	1 (3.8%)	8 (30.76%)	12 (46.2%)	2 (7.7%)	2 (7.7%)	1 (3.8%)

TABLE 4- MIC VALUES OF MBL PRODUCING A.BAUMANII TO MEROPENEM (n=12)

	MIC (µg/ml)							
	4	8	16	32	64	128	256	512
No. of Isolates	-	-	1 (8.3%)	4 (33.3%)	4 (33.3%)	2 (16.7%)	1 (8.3%)	-

TABLE 5- ANTIBIOTIC SUSCEPTIBILITY PATTERN OF MBL PRODUCING GRAM NEGATIVE BACILLI (n=98)

Antibiotic	E.coli (n=25)	Kpneumoniae (n=19)	P.vulgaris (n=6)	P.mirabilis (n=4)	K.oxytoca (n=4)	C.koseri (n=2)	P.aeruginosa (n=26)	A.baumanii (n=12)	Overall susceptibility (n=98)
AK	6 (24%)	3 (15.8%)	1 (16.7%)	1 (25%)	1 (25%)	1 (50%)	2 (7.7%)	3 (25%)	18 (18.4%)
GEN	5 (20%)	2 (10.5%)	2 (33.3%)	1 (25%)	1 (25%)	-	1 (3.8%)	2 (16.7%)	14 (14.3%)
CIP	2 (8%)	3 (15.8%)	2 (33.3%)	2 (50%)	1 (25%)	-	-	1 (8.3%)	11 (11.2%)
CZ	-	-	-	-	-	-	-	-	-
PT	-	-	-	-	-	-	-	-	-
IMP	2 (8%)	1 (5.3%)	-	-	1 (25%)	-	-	-	4 (4.1%)

FIGURE 1- MODIFIED HODGE TEST

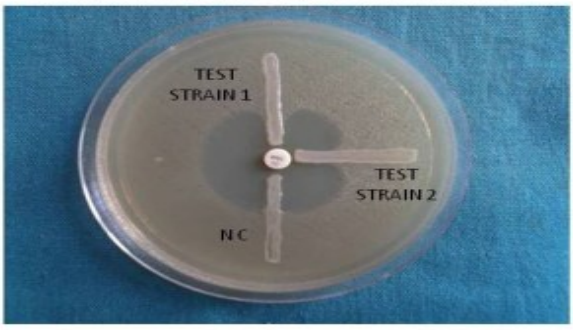

FIGURE 2- COMBINATION DISC TEST FOR DETECTING MBL PRODUCTION

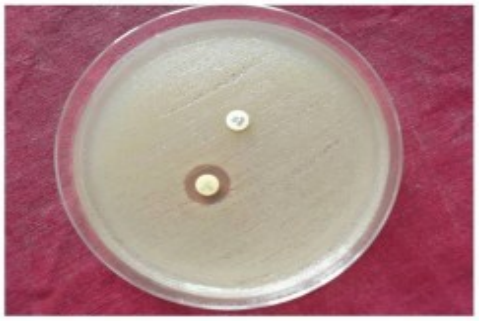

FIGURE 3- BROTH DILUTION METHOD FOR DETERMINING MIC TO IMIPENEM
(Test isolate showing MIC of 64µg/ml)

DISCUSSION

Carbapenemases are enzymes that hydrolyse carbapenems and are found amongst various species of gram negative bacilli. Ambler Class A serine carbapenemases occur predominantly among Enterobacteriaceae and are commonly mediated by KPC, IMI, SME, GES or NMC enzymes. They hydrolyse all lactams including 4th generation cephalosporins, cephamycins, monobactams and carbapenems but are inhibited by lactamase inhibitors. Class B enzymes are metallobetalactamases capable of hydrolyzing even lactamase inhibitors but not monobactams. The important enzymes implicated in MBL production are IMP, VIM, GIM, SPM and NDM. MBLs can be produced by *P.aeruginosa*, *Acinetobacter* species or Enterobacteriaceae. Class D serine oxacillinases are mainly produced by *Acinetobacter* species and hydrolyse all the above mentioned group of drugs.

In the present study, 3204 GNB were isolated in pure culture comprising of 2712 Enterobacteriaceae members, 305 *P.aeruginosa* and 187 *A.baumanii*. Of these, 1301 (47.9%), 188 (61.6%) and 98 (52.4%) respectively were found to be resistant to ceftazidime by disc diffusion method. Several studies have shown that ceftazidime resistance is a better indicator of carbapenemase production than imipenem resistance. So, the 1587 ceftazidime resistant isolates were subjected to Modified Hodge Test. MHT is a very sensitive and specific test for detecting the presence of carbapenemases.[5,6] Among 1301 ceftazidime resistant Enterobacteriaceae, 78 gave a positive MHT. However, when these were tested for MBL production using Combination Disc Test, only 60 gave a positive result. Since members of Enterobacteriaceae commonly produce Class A carbapenemases like KPC, it is likely that such isolates will fail to produce an increase in the zone size with EDTA as this enzyme is not Zinc dependent and thereby give a negative CDT. Further testing using 3-Amino phenyl boronic acid potentiation of meropenem disc test or CHROM agar KPC plate must be performed to confirm the presence of Class A carbapenemases. Among the 60 MBL producing Enterobacteriaceae, 25 *Escherichia coli*, 19 *Klebsiella pneumoniae*, 6 *Proteus vulgaris*, 4 each of *P.mirabilis* and *K.oxytoca* and 2 *Citrobacter koseri* were isolated. The results of MIC testing by broth dilution method showed that all of the MBL producing Enterobacteriaceae were resistant to imipenem with MIC $\geq$4 µg/ml. Most isolates exhibited MIC in the range of 16-32µg/ml.

Among the 188 ceftazidime resistant *P.aeruginosa* isolates, 26 were positive by both MHT and CDT. The prevalence rate of MBL producers observed was 8.5%. This is comparable to a study by Gupta *et al* where 7.5% of *P.aeruginosa* isolates were MBL producers.[11] All 26 MBL producing *P.aeruginosa* isolates were resistant to imipenem with MIC$\geq$ 16 µg/ml and 12 (46.3%) of them had MIC of 64µg/ml.

A total of 14 among 98 ceftazidime resistant *A.baumanii* isolates gave a positive MHT while only 12 gave a positive result on CDT. The resistant mechanism in the other 2 isolates could be possible production of other classes of carbapenemases like serine oxacillinases. Most of the MBL producing isolates exhibited MIC in the range of 32-64µg/ml.

In this study, 98 GNB isolates were found to be MBL producers comprising of 60 Enterobacteriaceae members, 26 *P.aeruginosa* and 12 *A.baumanii*. Highly significant statistical association ($p<0.0001$) was found between the isolate being a non- fermenting bacillus and the production of MBL (Table 1). Maximum numbers of MBL producing GNB were isolated from tracheal aspirates 37(37.8%), followed by urine 28(28.6%) and pus 23(23.4%). However, in a study from Maharashtra, maximum MBL producing GNB isolates were from pus followed by tracheal aspirates[12].

All the MBL producing isolates were resistant to most other classes of antibiotics as well. The genes that encode for MBLs are often found on Class I integrons. Other gene cassettes in the integron mediate resistance to other antibiotics like Aminoglycosides and Fluoroquinolones. These integrons are in turn embedded in transposons which due to their highly mobile nature facilitate interspecies transmission of these resistant enzymes. It was observed that 4 of the 98 MBL producing isolates in this study were sensitive to imipenem by disc diffusion method (Table 5). So, ceftazidime resistance was a more sensitive indicator of MBL production than imipenem resistance similar to the results of a study conducted in Nagpur[13]. However, the results of broth dilution method showed that these isolates were resistant to imipenem with MIC of 4µg/ml [3 isolates] and 8µg/ml [1 isolate] (Table 2).

This study showed varying prevalence of MBL production among different bacterial species ranging from 2.2% in Enterobacteriaceae to 8.5% in *P.aeruginosa*. The prevalence rate in *A.baumanii* was 6.4% and overall rate was 3.1%.This is much lower when compared to other studies in India.[14, 15]The lower prevalence rate may be attributed to lesser usage of carbapenems at our institution. Studies on MBL producing isolates in India have estimated the prevalence rate to vary between 7-38%.[14, 15] However, a recent study by Deshmukh *et al* showed 2.9% prevalence of MBL producing bacteria among GNB isolated at a tertiary care hospital in south India. [12]

CONCLUSION :

This study showed that various gram negative bacilli can produce metallobetalactamases. It is prudent to incorporate methods for detection of MBL production in the AST panel of GNB especially in tertiary care centres which often receive patients already on antibiotics and hence may harbor resistant organisms. The MHT is a sensitive test to detect the presence of carbapenemases and a simple test like CDT can detect MBL producing bacilli. In addition, MIC values must always be determined for isolates suspected to be MBL producers but appearing to be sensitive to imipenem in disc diffusion tests. These isolates may be actually resistant by broth dilution method. More surveillance studies to identify local resistance patterns like MBL production can aid in guiding empiric patient therapy and supplement the framing of antibiotic policy of the hospital.

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