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RESEARCH ARTICLE

DETECTION OF METALLO-B-LACTAMASE IN GRAM NEGATIVE BACILLI

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ABSTRACT

Metallo- β-lactamase (MBL) is an enzyme that makes bacteria resistant to a broad range of beta-lactam antibiotics. These include the antibiotics of the Carbapenem family, which are a mainstay for the treatment of antibiotic-resistant bacterial infections. The emergence of MBL in Gram negative bacilli (GNB) is becoming a therapeutic challenge worldwide. Detection of MBL is also a challenge for routine microbiology laboratories, since there are no standardized methods for MBL detection. The aims of this study were to know the prevalence of MBL production in various Gram negative bacilli, to evaluate different phenotypic methods to detect MBL production and to find out antibiotic sensitivity profile of MBL producing gram negative bacilli. (Nirav *et al.,* 2011) A total of 100 clinical isolates of GNB were subjected to antibiotic susceptibility testing. Ertapenem resistant clinical isolates were taken as positive for MBL screening. Three different phenotypic methods were used to confirm the MBL production: Ertapenem (ETP)-EDTA combined disc test, EDTA disc potentiation using Ceftazidime and Modified Hodge Test. Out of 100 clinical isolates of GNB, 48 isolates were resistant to Ertapenem. These 48 isolates were considered screening positive and further tested for MBL production by three different methods. 30 isolates were MBL positive by ETP-EDTA combined disc test and 23 isolates were MBL positive by EDTA disc potentiation using Ceftazidime and 19 isolates were positive by Modified Hodge test. In this study, 58.62% species of GNNF, 51.35% of *E.coli*, 41.67% of *Klebsiella pneumonia* and 20% of *Proteus mirabilis* were MBL positive. The detection of MBL-producing isolates is crucial in GNB isolates and ETP-EDTA combined disc test is the most effective method.

INTRODUCTION

The mechanisms of bacterial resistance are complex, varied and not completely understood. In the past two decades, clinical microbiologists have been finding it difficult to test the antibiotics against many of the common pathogens. The reason being the emergence of increasing resistance to the betalactam group of drugs which include the carbapenems, apart from the indiscriminate use of antibiotics. The carbapenemases are diverse enzymes that vary in their abilities in hydrolyzing carbapenems and other betalactams. Hence, their detection is a crucial issue because they often show an extensive and sometimes a total antibiotic resistance. The plasmid mediated carbapenemases pose more danger than the chromosomal mediated carbapenemases. The carbapenemases belong to the molecular classes, A, B and D. The class B enzymes (Bush group 3) are MBLs. The MBLs hydrolyze almost all the betalactam antibiotics. Several phenotypic methods are available for the detection of the MBLs which are produced by bacteria. Most of these methods are based on the ability of the metal chelator (EDTA) and the thiol based compounds to inhibit the enzyme activities. The occurrence of an MBL positive isolate in a hospital environment poses not only a therapeutic problem but also a serious concern for infection control management. Thus, in order to control the spread of resistance, the detection of MBL is of prime importance. (Noyal *et al.,* 2009)

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MATERIALS AND METHODS

The current prospective study of 100 Gram negative bacilli from various clinical samples (sputum, pus, urine, blood, and other samples) processed in the Department of Microbiology, Victoria Hospital, BMCRI over a period of 2 months during March and April 2013. Identification was done by standard biochemical tests. Antimicrobial susceptibility testing was done according to Clinical Laboratory Standards Institute (CLSI) recommended Kirby-Bauer disk diffusion method. A lawn of the test organism was made on the Mueller-Hinton Agar (MHA) after adjusting the inoculum to 0.5McFarland unit and discs are placed by novel disc placement pattern and incubated at 35^0C for 18-24h in ambient air. The following antibiotics were tested by the disk diffusion method: Piperacillin (100 µg),

Piperacillin/Tazobactam (100 µg/10 µg), Ceftazidime (30 µg), Cefepime (30 µg), Cefoperazone (30 µg), Ceftriaxone (30 µg), Cefotaxime (30 µg), Amikacin (30 µg), Ciprofloxacin (5 µg) and Ertapenem (10 µg).

The isolates which were found to be resistant to Ertapenem were considered as suspected MBL producers and following tests were done to confirm the same. The different phenotypic confirmatory methods as per CLSI guidelines are (Noyal *et al.,* 2009):

Ertapenem (ETP)-EDTA combined disc test

Test organisms were inoculated on to plates with Mueller Hinton agar as recommended by the CLSI. Two 10 µg Ertapenem disks (Becton Dickinson) was be placed on the plate, and appropriate amounts of 10 µL of EDTA solution was added to one of them to obtain the desired concentration (750 µg). The inhibition zones of the Ertapenem and Ertapenem-EDTA disks were compared after 16 to 18 hours of incubation in air at 35°C. In the combined disc test, if the increase in inhibition zone with the Ertapenem and EDTA disc is ≥ 7 mm than the Ertapenem disc alone, it will be considered as MBL positive.

EDTA disc potentiation using Ceftazidime, Ceftizoxime, Cefepime and Cefotaxime

Test organisms were inoculated on to plates with Mueller Hinton agar as described for the standard disc diffusion test. A filter paper (Whatmann No. 2) blank disc was placed and the following discs [Ceftazidime (30 µg), Ceftizoxime (30 µg), Cefotaxime (30 µg), Cefepime (30 µg)] was placed 25mm center to center from the blank disc. Ten microlitre of 0.5 M EDTA solution was added to the blank disc and the plate was incubated overnight at 35º C. Enhancement of the zone of inhibition in the area between the EDTA disc and any one of the four cephalosporin discs in comparison with the zone of inhibition on the far side of the drug was interpreted as a positive result (Behera *et al.,* 2008)

Modified Hodge test

E. coli ATCC25922 was inoculated after it is made into an inoculum of 0.5 McFarland units and diluted in 1:10 parts on to a MHA plate. Allow the plate to dry 3 to 10 minutes. A 10 µg Ertapenem disk was placed at the centre of the plate and using a 10-µL loop or swab, 3 to 5 colonies of test organism grown overnight on a blood agar plate were inoculated in a straight line out from the edge of the disk. The streak was at least 20 to 25 mm in length. It was incubated overnight. The presence of a distorted zone of inhibition was interpreted as a positive result. (Clinical and laboratory standard institute)

RESULTS

In the present study, 100 isolates of Gram negative bacteria were obtained from various clinical samples like pus, sputum, blood and urine and were screened for susceptibility to various antibiotics. MBL detection and confirmation was done.

The results obtained have been elaborated below.

Of the 100 clinical isolates of Gram negative bacteria screened according to CLSI guidelines, 48 were selected for confirmatory tests for MBL.

Few studies have reported that 'Imipenem-EDTA combined disk test' as the most specific method for detection of MBL production in Gram negative bacilli (Pandya *et al.,* 2011; Franklin *et al.,* 2006). Hence we followed the combined disk method using Ertapenem- EDTA as the gold standard for the detection of MBL in the Gram negative organisms. 30 test samples (62.5%) were found to be MBL positive by this method.

These samples were tested for MBL production by other phenotypic methods such as EDTA disc potentiation using Ceftazidime and Modified Hodge test. By the EDTA disc potentiation test using Ceftazidime, 76.67% were MBL positive isolates (23 out of 30 MBL positive).

In Modified Hodge test, a positive result was observed in 63.33% MBL isolates (19 out of 30 MBL positive).

Table 1. Comparison of all tests

	MBL positive by screening method	ERT – EDTA combined disc test	Modified Hodge test	Disc potentiation test
GNNF	17	13	9	10
E.coli	19	9	6	7
K.pneumoniae	10	7	3	5
P.mirabilis	2	1	1	1

Table 2. Comparison of modified HODGE test with ERT-EDTA combined DISC test

	ERT – EDTA combined disc test	Modified Hodge test	% detected as MBL positive by Modified Hodge test
GNNF	13	9	69.2
E.coli	9	6	66.67
K.pneumoniae	7	3	42.86
P.mirabilis	1	1	100

Table 3. Comparison of disc potentiation test with ERT-EDTA combined DISC test

	ERT – EDTA combined disc test	Disc potentiation test	% detected as MBL positive by Disc potentiation test
GNNF	13	10	76.92
E.coli	9	7	77.78
K.pneumoniae	7	5	71.4
P.mirabilis	1	1	100

The phenotypic methods used in the study were Ertapenem – EDTA combined disc test, Modified Hodge test and EDTA disc potentiation test using Ceftazidime. The study showed

Disc potentiation test is superior to Modified Hodge test. The former test confirmed 23 out of 30 while the latter confirmed 19 only.

The sample wise distribution of the MBL positive gram negative bacteria is shown in Figure 1. The maximum number of MBL producers were obtained from Pus samples-41.67%, followed by Urine-27.08% , Endotracheal tube-10.42%, Blood and Sputum -8.33% each and Oral swab and Umbilical catheter tip- 2.08% each.

In the present study, 58.62% species of GNNF, 51.35% of *E.coli*, 41.67% of *Klebsiella pneumoniae* and 20% of *Proteus mirabilis* were MBL positive. The species distribution is shown in Figure 2.

The antimicrobial susceptibility patterns of the 48 MBL producers revealed that a high rate of resistance was found for Aztreonam, Ceftazidime (87.5%), to Ciprofloxacin (62.5%), to Pipracillin/Tazobactam (50%) and to Amikacin, Cefepime (25 %). The study also revealed Tigeycline was the most effective drug against MBL producing isolates as only 16.66% isolates showed resistance to this drug. The resistance pattern is shown in Figure 3.

DISCUSSION

The introduction of carbapenems for treating serious bacterial infections which were caused by the β- lactam resistant bacteria was a great leap in the antibiotic history.

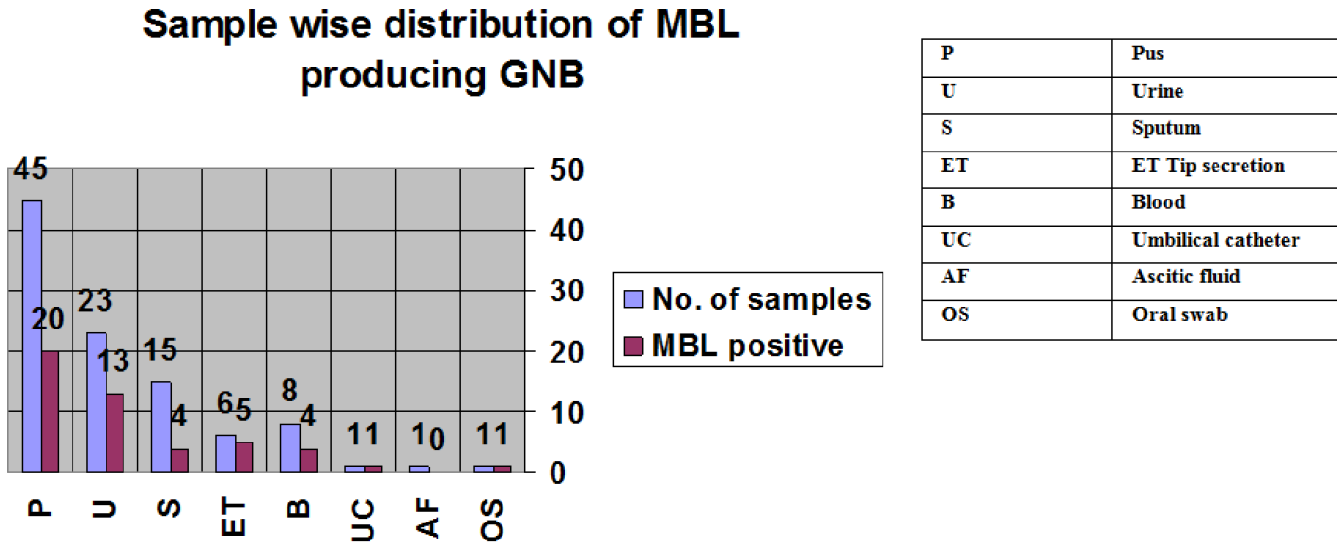

Fig. 1. Sample wise distribution of MBL producing GNB

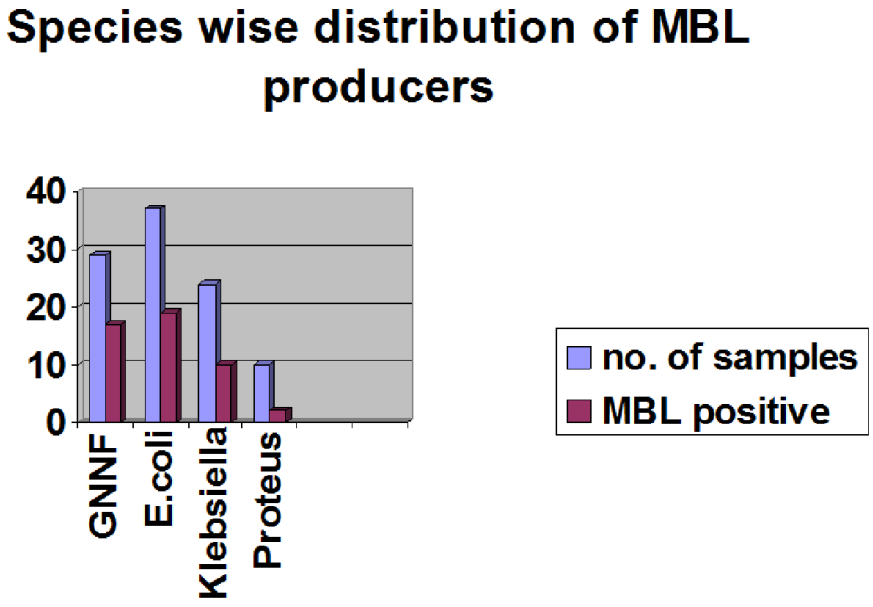

Fig. 2. Species wise distribution of MBL producers

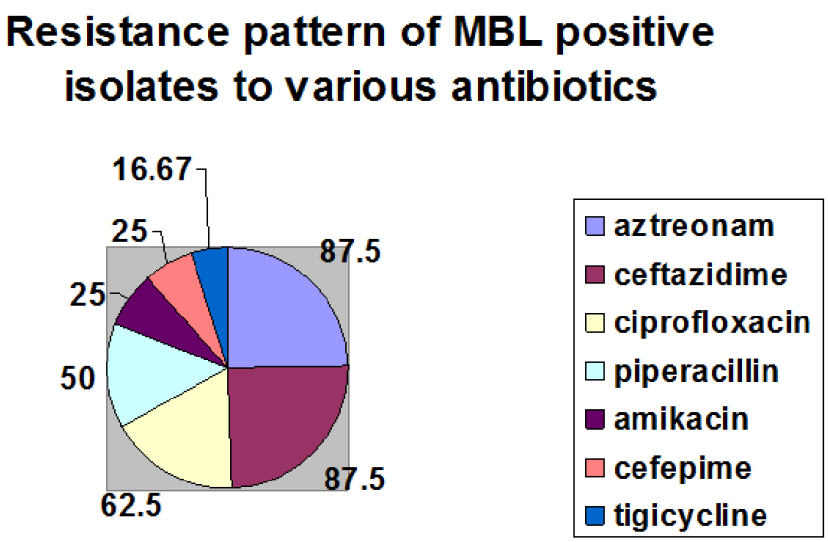

Fig. 3. Resistance pattern of MBL positive isolates to various antibiotics

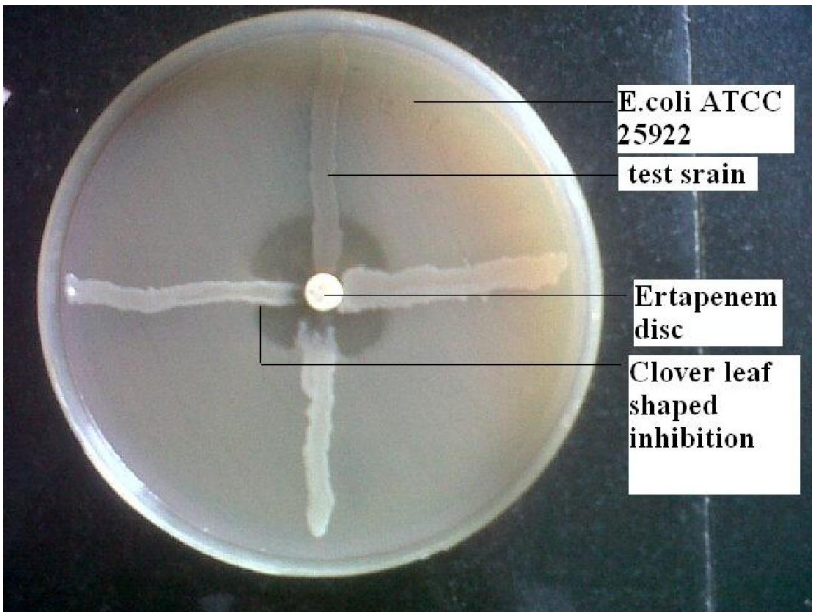

Fig. 4. Modified Hodge test. Positive strain shows 'cloverleaf shaped' zone of inhibition due to carbapenemase production, while the negative strain shows an undistorted zone of inhibition

But certain organisms have evolved in such a way that the effect of these carbapenems no longer seem to be very promising. Two types of carbapenem hydrolyzing enzymes have been described –the serine enzymes which have a serine moiety at the active site and MBLs which require divalent cations like zinc as a cofactor for their enzymatic activity . However, these enzymes are universally inhibited by EDTA and other chelating agents of the divalent cations. Several non-molecular methods have been studied for the identification of the carbapenemases and in many cases; their detection is based on the use of specific inhibitors. Hence, the MBL detection is based on its dependence on zinc and on the use of inhibitors/chelating agents such as EDTA. (Prakash *et al.,* 2012)

Since there are no standard guidelines for detection of MBL, different studies have reported the use of different methods.

PCR analysis is the gold standard method for the detection of MBL production, but it is not feasible in routine microbiology laboratory. (Nirav *et al.,* 2011) In present study, out of 100 Gram negative bacilli isolates, 48 isolates were screened as positive for MBL production. ETP- EDTA combined disc test detected 30 MBL positive isolates and EDTA disc potentiation test detected 23 MBL positive isolates whereas Modified Hodge test detected 19 MBL positive isolates. The present study indicated that the former 2 tests are more superior to the latter. The result is consistent with other similar studies, though the reason for the difference in the performance of these tests is not clear. The reason for the increased sensitivity of EDTA-Ceftazidime combination is the ability of Ceftazidime to produce a marked inhibitory effect with EDTA. Therefore, Ceftazidime appears to be the better substrate for EDS. Similar results were observed in a study done at the National Institute of Infectious Diseases, Tokyo, and Japan. (Noyal *et al.,* 2009)

Carbapenem hydrolyzing enzymes are most commonly seen in nonfermenter gram negative organisms (non enterobacteriaceae) i.e. *Pseudomonas* and *Acinetobacter*. However, in the recent years there is an increasing incidence of these enzymes in Enterobacteriaceae family as well (Balan *et al.,* 2012).

In the present study, higher rates of MBL production was seen in Gram negative nonfermenters which included *Pseudomonas* and *Acinebacter* species followed by Enterobacteriaceae *E.coli and K.pneumoniae.* This is consistent with results of studies by Pandya *et al.* (2011) 62.5% of the Ertapenem resistant Gram negative bacterial isolates were MBL positive in our study. Kaleem *et al.* 2010 in Pakistan reported 78 % of the Gram negative bacterial isolates as MBL positive.

The detection of MBL and other carbapenemases is of utmost importance in deciding the most appropriate therapeutic regimen for treatment of the Carbapenem resistant nonfermenters. In several studies, intravenous Colistin combined with Rifampin and Imipenem was recommended for the treatment of Carbapenem-resistant isolates lacking MBLs, whereas the combination of Colistin and Rifampicin (with or without Tigecycline) was recommended for treatment of MBL producing carbapenem-resistant isolates. Detection of carbapenemases is necessary for administration of appropriate therapy.

The drawbacks in our study are the relatively small sample size and failure to evaluate the clinical usefulness of detection of carbapenemases. Our study was restricted mainly to detection of carbapenemases and the comparison of the efficacy of different techniques for detection of carbapenemases. Further studies are needed to evaluate the clinical usefulness of detection of carbapenemases. (Noyal *et al.,* 2009) To conclude, the high rate of MBL producing Gram negative bacteria in this study emphasizes on the need for active surveillance in the microbiology labs for the detection of these resistant strains and also stresses on the judicious use of carbapenems to prevent the spread of resistance. Different phenotypic methods for detection of these carbapenemases are available, but controversies exist regarding the choice of optimal laboratory method. Microbiology laboratories must be prepared to screen for MBL-producing isolates by a low cost, convenient and sensitive procedure. (Sangeetha *et al.,* 2014)

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