Original Article

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Microbiology Section

The Prospective Evaluation of Four Convenient Methods for Detecting MBLs in the Clinical Isolates

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ABSTRACT

Background: The emergence and the spread of metallo-beta-lactamases (MBLs) in non fermenters like Pseudomonas aeruginosa and the Acinetobacter spp has become a therapeutic challenge. In this study, the phenotypic confirmation of the MBL production was done by different methods.

Method: The isolates were screened for metallo-beta-lactamase production by using imipenem. All the imipenem resistant isolates were further subjected to a phenotypic confirmation by the combined disk method, the double disk synergy test and the modified Hodge test. The results were analyzed and tabulated.

Results: Of the total 200 isolates, 124(62%) were of P. aeruginosa and 76(38%) were of the Acinetobacter spp. Out of the 200 isolates, 28 (19(68%)- P. aeruginosa and 9(32%)- Acinetobacter isolates were found to be MBL producers by the combined disk test which used imipenem and EDTA , by the double disk synergy test and by the modified Hodge test. But the combined disk test which used ceftazidime detected only 25 out of the 28 MBLs.

Conclusion: The results of our study showed that the combined disk test which used imipenem, the double disk synergy test and the modified Hodge test, all had equal sensitivity. But when the ease of the performance and the interpretation of the results were considered cost effectively, the combined disk test was found to be a more feasible option than the other tests. Overall, these tests can be done easily as they are less laborious and are cost – effective. Hence, the routine testing of the metallo beta-lactamase producers is of great value, in order to take measures to advocate a proper antibiotic policy and also to take effective steps for controlling their spread worldwide.

Key Words: MBLs, Pseudomonas aeruginosa, Imipenem-EDTA combined disk test, Double disk synergy test, Modified Hodge test.

INTRODUCTION

The mechanisms of bacterial resistance are complex, varied and not completely understood [1]. In the past two decades, the clinical microbiologists have been finding it difficult to test the antibiotics against many of the common pathogens.The reason being the emergence of the increasing resistance to the betalactam group of drugs which include the carbapenems, apart from the indiscriminate use of antibiotics.

The carbapenems (imipenem, ertapenem and meropenem) are the mainstay of treatment for serious infections which are caused by non fermenting bacilli like Pseudomonas aeruginosa, Acinetobacter spp, etc.

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 more resistant organisms like the strains of Pseudomonas and Acinetobacter spp. have acquired the resistance from the Enterobacteriaceae [2]. 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 metallo betalactamases (MBLs). The MBLs hydrolyze almost all the betalactam antibiotics. The MBLs typically hydrolyze carbapenem efficiently, but they are inhibited by chelating agents such as EDTA.

The MBL activity can be detected by both phenotypic and genotypic methods. Different studies have used different methods according their feasibility. Though the molecular methods are highly sensitive and specific, their use is limited only to the research laboratories. The phenotypic methods being simple, sensitive, economical and reliable, they can be routinely performed in microbiological laboratories. 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 [3].

The spread of the MBL genes from Pseudomonas aeruginosa to Enterobacteriacea is posing a difficulty in selecting the antibiotics for controlling serious infections like septicaemia, pneumonia, etc,. Thus, in order to control the spread of resistance, the detection of MBL is of prime importance. This study was aimed at detecting the MBL producing Pseudomonas aeruginosa as well as members of the Enterobacteriaceae family.

MATERIALS AND METHODS

The study period and the clinical samples - A total of 200 samples (from the patients who were admitted to the Bapuji and the Chigateri Government Hospital, Davangere) which included blood, urine,

pus , wound swabs , suction tips, catheter tips and other body fluids were included in this study. This study was carried out over a period of 11 months (from Jan 2011 to Nov 2011).The samples were processed as per the standard microbiological procedures [1]. Pseudomonas aeruginosa was identified, based on its colony morphology on blood agar and Mac Conkey's agar, the oxidase test , its pigment production and polymyxin B (300 units) on the Muller Hinton agar (MHA).

Antimicrobial susceptibility testing- Antibiotic susceptibility testing was performed on Mueller Hinton Agar (Hi Media) by the Kirby-Bauer disk diffusion method according to the CLSI guidelines (2006). The antibiotic disks for the studies were procured from Hi Media, Mumbai, India. The drug disks which were tested in our study were, (in micrograms)- Gentamicin (10), Amikacin (30), Ciprofloxacin (30), Ceftazidime (30), Ceftriaxone (30), Imipenem (10), Polymyxin B (300), Piperacillin-Tazobactum (100/10), Cefotaxime (30) and Cefepime (30). The isolates which showed a zone diameter of ≤13mm were considered as resistant, those which showed a zone diameter of 14-15mm were considered as intermediate and those which showed a zone diameter of ≥16mm were considered to be sensitive to imipenem. The isolates which showed resistance to imipenem were further subjected to the MBL phenotypic confirmation.

Pseudomonas aeruginosa ATCC 27853 and E. coli ATCC 25922, were used as the control strains, which were obtained from HI-Media, Mumbai.

The different phenotypic confirmatory methods which were used were as follows:

1. The Imipenem –EDTA combined disk method – A 10μg Imipenem disk was placed on a Mueller Hinton agar plate at a distance of 20mm from an Imipenem-EDTA disk, on a lawn culture of an imipenm resistant isolate. The plates were incubated overnight at 37^{0}C and the zone of inhibition of the imipenem and the imipenem -EDTA disks were compared on the next day. If the increase in the inhibition zone with the Imipenem-EDTA disk was ≥ 7mm than the imipenem disk alone, it was considered to be MBL positive.

2. The Ceftazidime - EDTA combined disk method - Two 30μg ceftazidime disks were placed on a Muller Hinton agar plate, on which the lawn culture of an imipenem resistant isolate was made. 10μl of EDTA solution was added to one of them, to obtain the desired concentration of 750μg. The plates were incubated overnight at 370C and the zone of inhibition of the ceftazidime and the ceftazidime EDTA disks were compared on the next day. If the increase in the inhibition zone with the ceftazidime-EDTA disk was ≥ 7 mm than the ceftazidime disk alone, it was considered to be MBL positive.

3. The Imipenem- EDTA double disk synergy test (DDST) – A 10 μg imipenem disk was placed at 20 mm centre to centre from a blank disk which contained 10ul of 0.5 molar EDTA (750 μg). After an overnight incubation at 370C, the enhancement of the zone of inhibition around the imipenem EDTA disk in comparison with the zone of inhibition on the far side of the drug was interpreted as positive for MBL production.

4. The Modified Hodge test - E. coli ATCC25922 was inoculated on to a MHA plate as per the CLSI guidelines. The test organisms were heavily streaked from the centre to the periphery of the plate. The plate was allowed to stand for 15 minutes at room temperature. A 10 μg imipenem disk was placed at the centre of the plate and it was incubated overnight. The presence of a distorted zone of inhibition was interpreted as a positive result.

RESULTS

Out of the 200 samples which were collected, 112(56%) were from males and 88(44%) were from females. Of the 200 samples, 124(62%) were of Pseudomonas and 76(38%) were of Acinetobacter. 36(18%) of them showed resistance to Imipenem (screen positives). But when they were subjected to various phenotypical confirmatory methods, we found that a total of 28 (78%) were MBL producers. This result indicated that 14% of all the isolates (200) were MBL positives. The details of the organisms and the MBL production and the methods which were used have been shown in the tables below [Table/Fig-1], [Table/Fig-2], [Table/Fig-3] and [Table/Fig-4].

Organism	No,%	MBL producers(28)
Pseudomonas aeruginosa	124(62%)	19(68%)
Acinetobacter spp	76(38%)	9(32%)

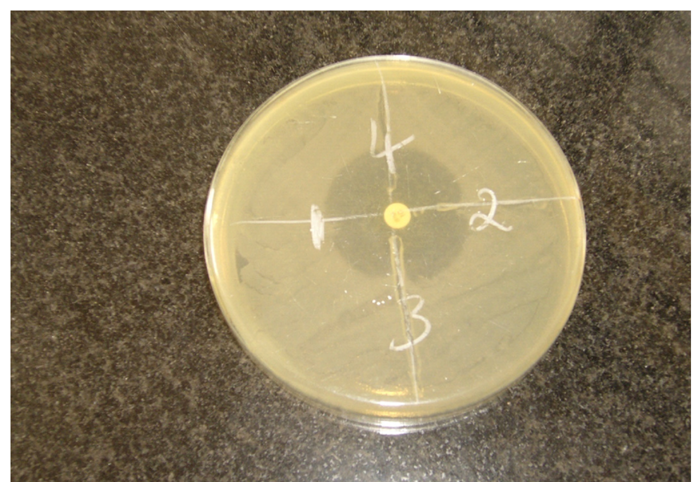
[Table/Fig-1]: Organism wise distribution of MBL producers

Source	No	MBL positive No, %
Urine	81	4, (14%)
Pus	51	13, (46%)
Blood	23	1, (4%)
Sputum	34	2, (7%)
ET/ST	11	8, (29%)
TOTAL	**200**	**28(14%)**

[Table/Fig-2]: No and % of MBL positives in various samples

Method	Pseudomonas (19, 68%)	Acinetobacter (9, 32.%)
CDT(CA-EDTA)	17(60.71%)	8(28.57%)
CDT(I-EDTA)	19(67.85%)	9(32.14%)
DDST(I&EDTA)	19(67.85%)	9(32.14%)
MHT	19(67.85%)	9(32.14%)
Total (28)		

[Table/Fig-3]: Showing MBL positives by different methods



[Table/Fig-4]: Photograph showing Modified Hodge Test. Distorted zone of inhibition is seen around the Imipenem disk at the center

DISCUSSION

Pseudomonas aeruginosa which produces metallo-β- lactamases (MBLs) was first reported from Japan in 1991 [4], and

it has spread worldwide since then. Apart from P.aerugenosa, other bacteria like Serratia, Klebsiella pneumonia , Escherichia coli, Enterobacter aerogens, E.cloacae, Citrobacter freundii, Proteus vulgaris, P.putida, Acinetobacter and Alcaligenes xylosoxidans were also shown to produce MBLs [5]. 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 [6]. 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 Metallo-β-Lactamases (MBLs) which require divalent cations like zinc as a cofactor for their enzymatic activity [7-10]. The serine carbapenemases are the derivatives of the class A or the class D enzymes and they usually mediate carbapenem resistance in Enterobacteriaceae or in the Acinetobacter spp [11]. Despite the high avidity of these organisms for the carbapenems, they do not always mediate high-level resistance and not all are inhibited by clavulinic acid [12]. The classification scheme which was proposed by Ambler in 1980 was modified, depending on the functional properties of the carbapenems, by Bush in 1989.This has been a widely accepted referencing system for the β-lactamases .This was mainly based on their Imipenem hydrolysis, their sensitivity to EDTA and their lack of inhibition by the serine β-lactamase inhibitors. This scheme was further updated in 1994 and 1997 to accomodate the increasing new enzymes which were continually being discovered [13,9,14]. Class B1, B2 and B3 were the chromosomally mediated MBLs, whereas the transferable MBLs included IMP, VIM, GIM and SPM-1 [15]. However, these enzymes are universally inhibited by EDTA and other chelating agents of the divalent cations [14]. 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 [15,16].

Since there are no standardized detection methods, many researchers have used many different methods to detect and to confirm the MBL production. In our study, we used the combined disk test, the double disk synergy test and the modified Hodge test. In the present study, out of 124 P. aeruginosa isolates, 19(15.32%) and out of 76 Acinetobacter isolates, 9(11.84%) were found to be MBL positives. Hence, out of 200 isolates, a total of 28(14%) were MBL positive. The details have been shown in [Table/Fig-3].

The Combined Disk Test (CDT) which used ceftazidime (CAZ) and EDTA detected 17(60.7%) out of 19 isolates of Pseudomonas and 8(28.5%) out of 9 isolates of Acinetobacter. But on using imipenem and the I- EDTA combined disk test as was described by Yong et al., [5] all the 28(14%) isolates were found to be MBL producers. This result was similar to that of the study which was done by Deeba Bashir et al., where they found 12% of the P. aeruginosa isolates to be MBL producers by CDT [17]. But in another study which was done by Horeish Saderi et al,. 39.06% of MBLs were observed. In a study which was done by Noyal et al,. 6.5% of the Acinetobacter isolates were found to be MBL producers . The DDST in our study also confirmed all the 28 isolates to be MBL producers. When they were subjected to the modified Hodge test again, all the 28 isolates were found to be confirmed MBL positives. Three different methods (CDT, DDST, MHT) gave consistent results, whereas a slightly different result was given by CDT which used ceftazidime. The reason could be that the MBL producing organisms had other ceftazidime resistance mechanisms. Such strains will not show MBL production [18]. Many, including Arakawa et al,. have suggested that the combined disk test was superior to the double disk synergy test. The major drawback of DDST was the subjective interpretation of the results at times [19]. But our study showed both of them to be equally effective in detecting the MBLs. It has been suggested that the selection of the optimal MBL screening method be based not only on the bacterial species, but also on the strains which are collected and the local prevalence of the MBL producers [20,21].

When all the 28 isolates were tested for their susceptibility patterns by using the Mueller Hinton Agar, both Pseudomonas and Acinetobacter showed 94%, and 96% resistance to the fluoroquinolones respectively and 92% and 100% resistance to the aminoglycosides. An increased level of resistance was also seen by Prajapathi et al,. [22]. In our present study, we also noted that the isolates showed 78% resistance to cefepime and 61% resistance to piperacillin-tazobactum. A similar pattern was observed by Behera et al,. With the MBLs showing an increased resistance pattern to a variety of commonly used and effective drugs, one is left with a very minimal choice of drugs for the treatment of the severe infections which are caused by these organisms.

CONCLUSION

To conclude, the results of our study showed that the combined disk test and the double disk synergy test were equally effective and that they were at par with the modified Hodge test which was commonly used as a final confirmatory test, apart from the E-test and molecular based methods. Many studies have proved that the MBL E-test and the combined disk test had equal sensitivity. As the E-test is highly expensive, CDT, which is rather economical and easy to do, can be routinely implemented. DDST and MHT can also be used to corroborate the results .Our study results give a warning regarding the serious scenario of the MBLs. This also points towards the implementation of a strict intra-institutional antibiotic policy and infection control measures to limit the spread of these MBLs globally.

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