Indian Journal of Medical Microbiology, (2011) 29(3): 302-4

Brief Communication

Metallo beta lactamase producing *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

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Abstract

This study aims in identifying MBLs particularly Zn requiring Molecular Class B enzymes produced by *Pseudomonas aeruginosa* and *Acinetobacter baumannii* .The resistance by these organisms are in a rise against all antibiotics including carbapenems and no prescribed CLSI guidelines is available for detecting them. Clinical isolates antibiotic susceptibility was determined by number of phenotypic tests by addition of 50mM of 10 µl zinc as cofactor for metallo beta lactamase production along with 0.5M ETDA of 5µl (930 µg per disk) plain disks. Increase in zone size of the meropenem -EDTA disk compared to the meropenem disk without EDTA was recorded positive. For Zn requiring MBLs zone towards both disks of EDTA and Zn along with meropenem is detected by DDST.

Key words: *Metallo-b-lactamases, MIC of meropenem, zinc substitution*

Introduction

The leading cause of resistance to β-lactam antibiotics like penicillin, cephalosporin, cephamycin and /or monobactam among gram negative bacteria is due to extended-spectrum β- lactamase produced by these organisms they inactivate the antibiotics by hydrolysing the β-lactam ring. Most ESBL producing organisms are in the family Enterobacteriaceae commonly in *E.coli* and *Klebsiella pneumonia*, but *Acinetobacter* and *Pseudomonas* are also reported. The high resistance for antibiotics and the rapid dissemination of metallo beta lactamase producing isolates in hospitals require an effective phenotypic method for identifying them as there are no prescribed CLSI guidelines available for detecting them. Molecular class B enzymes have zinc in their active site[1] so the catalytic activity of this enzyme can be enhanced for detection by incorporating it for the tests, unlike serine carbapenem resistance they are not inhibited by clavulanic acid, sulbactam and tazobactam.[2] This study aims to provide an early, rapid and effective phenotypic method for identifying MBLs in hospitals especially in processing ICU samples for carbapenems drug resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* which is being increasingly reported.

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Received: 28-03-2011
Accepted: 27-06-2011

Access this article online	
Quick Response Code:	Website: www.ijmm.org
	DOI: 10.4103/0255-0857.83918

Materials and Methods

Isolates of MDR gram negative organisms over a period of one year that showed decreased susceptibility to oxyimino-cephalosporins were tested phenotypically as per CLSI guidelines. Non repetitive ceftazidime resistant Enterobacteriaceae (*E.coli, Klebsiella* spp*, Enterobacter* spp), *Acinetobacter* spp*, and Pseudomonas spp* that were frequently isolated from samples of urine, blood, sputum, pus, endotracheal aspirates, bronchial secretion, wound and vaginal swabs were selected for the study. Antimicrobial susceptibility testing was initially determined with cephalosporins, aminoglycosides, penicillins and carbapenems (Hi-Media). Zone diameter was measured and interpreted as per CLSI guidelines for quality control of disc diffusion tests ATCC *E.coli* 25922 and an in-house carbapenem resistant *Acinetobacter baumannii* were used and they showed the expected zone pattern. The test isolates opacity was adjusted to 0.5 McFarland's standard and a lawn was spread on Mueller Hinton agar plates for all the tests.

Test for ESBLs Production

Disk approximation method[3]

Isolates found resistant or with decreased susceptibility to any one of the third generation cephalosporin antibiotics were selected as ESBLs producers. All ESBL producers showed enhanced zone diameter of 5 mm or greater for ceftazidime –clavulanic acid with respect to ceftazidime alone. The carbapenem MDR resistant *Acinetobacter spp* and *Pseudomonas spp* were further screened for MBL production.

Test for Metallo β-lactamase Detection

Disk potentiation test[4]

Two 10µg meropenem disks were placed on inoculated plates wide apart, to one of the disk10 µl of 50mM zinc sulphate was added after drying, 0.5M ETDA of 5µl (930 µg per disk) was then dispensed.EDTA (Sigma Chemicals) of pH 8 was prepared with distilled water and sterilized by autoclaving. After overnight incubation, an increase in zone size of ≥ 7mm around the meropenem -EDTA disk compared to the meropenem disk alone was recorded positive.

Double disk synergy test[5]

This test was performed with an overnight broth culture of the test strain inoculated on the MHA plate and allowed to dry. 5µl of the EDTA solution was added to a 6-mm blank filter paper disk (Whatman filter paper) which contained approximately 930 µg of EDTA. A 10 mg meropenem disk was placed in the centre of the plate flanked by EDTA disk and 10 µl of 50mM zinc sulphate disk [Figure 2] at a distance of 15 mm.After overnight incubation, the presence of an enlarged zone of inhibition towards the EDTA disk was interpreted positive for an MBL producer and an extended zone towards Zn for the putative ambler class B MBLs.

Modified hodge test[5]

Modified Hodge test was done although not recommended for MBL detection (MHT has been originally described by the Centre for Disease Control for Carbapenemases detection in Enterobacteriaceae) with carbapenem sensitive ATCC *E.coli* 25922 spread on the MHA plate. A 10 µg meropenem disk was placed in the centre of the plate and 10 µl of 50mM zinc sulfate solution was added to the disk along with EDTA. Carbapenem resistant NLF isolates were streaked with good inoculum of 0.5cm around the meropenem starting from the disk to outwards in four different directions. The plates were incubated at 37°C overnight; zone around the meropenem disk with clover leaf indent is taken as positive test.

MIC of Meropenem by Broth Microdilution Method[6]

Strains found resistant to meropenem by the disk diffusion test were tested by CLSI broth microdilution method using Mueller-Hinton broth. Doubling dilutions for meropenem ranging from 0.25 µg/mL though 64 µg/mL were tested. Meropenem powder for injection (Astra Zeneca, U.K. Ltd.) was used for the test. ATCC *Pseudomonas aeruginosa* 27853 was used as the control strain. MIC of 16 µg /mL or above was interpreted as resistant to meropenem.

Results

The ESBL isolates includes *E.coli* 63.5%, *Enterobacter spp* 66.3% and *Klebsiella spp* 56.1% these resistant isolates are treatable with available β –lactam inhibitors. MBL was positive in 36 of 242 (14.8%) of *Acinetobacter baumannii and* 92 of 331 (27.7%) of *Pseudomonas aeruginosa*. As a result of widespread application of antimicrobial agents the frequency of drug resistance to *Pseudomonas* is raising dramatically[7] imipenem resistance was77.5%, but from literatures[8] the resistance pattern for imipenem was 26%. *Acinetobacter spp* imipenem resistance 25.6% had been reported[9] but in this study we find 99% resistance. *Acinetobacter spp* accounting for 86% of resistance[10] among all the multidrug resistant isolates as shown in [Figure 1] was sensitive only for tigecycline and colistin. MIC determined by the broth microdilution method for the MBL positive isolates showed low-level resistance or sensitivity to meropenem. Taking an MIC of 16 µg/mL or above as resistant to meropenem, five isolates obtained from twelve patients, was 64 µg/mL and found to have MICs to meropenem in the sensitive zone. The remaining isolates showed MICs of 0.25-0.5 µg/mL. Meropenem disks incorporated with EDTA and Zn in other two tests is not clear for interpretation although an increase in zone size of 3 to 4mm was observed in comparison with meropenem alone may be due to EDTA.Few strains showed synergy with EDTA and Zn, suggesting that putative DDST possibly identifies Ambler ClassB MBLs.

Discussion

The horizontal spread of resistance factors into environmental gram negative bacteria has seen the emergence of multidrug resistant *Acinetobacter, Pseudomonas*, *Serratia*, *Stenotrophomonas spp*.A few tested *Klebsiella spp* showed improved zone in MHT with zinc sulfate although reports of no significant improvement was shown.[11] AmpC detection was done on few MBL negative *Acinetobacter spp* but the number is not substantial to furnish, along with MIC for colistin and efflux mechanism. Among all the tests used for MBL detection DDST at 15mm distance is more reliable to CDT and MHT, sensitivity was also clear when zinc sulfate was dispensed to the disks as cofactor for metallo beta lactamase production. Plain disks with 50mM zinc sulfate and 0.5M EDTA at 15mm from meropenem on either sides (instead of adding to the meropenem disk) showed synergy with both the disks(Zn and EDTA) as in [Figure 2] a putative

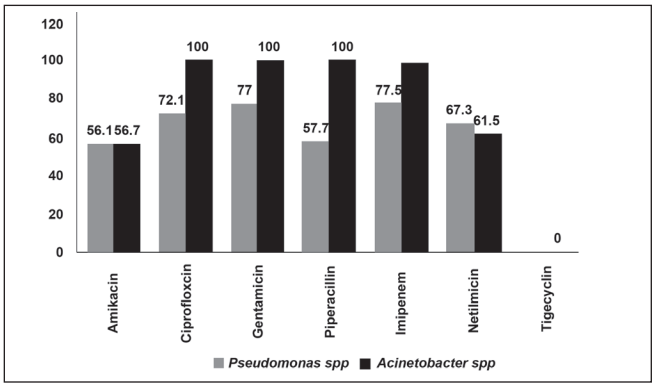

Figure 1: Resistance percentage of *Acinetobacter spp* and *Pseudomonas spp* for individual antibiotics

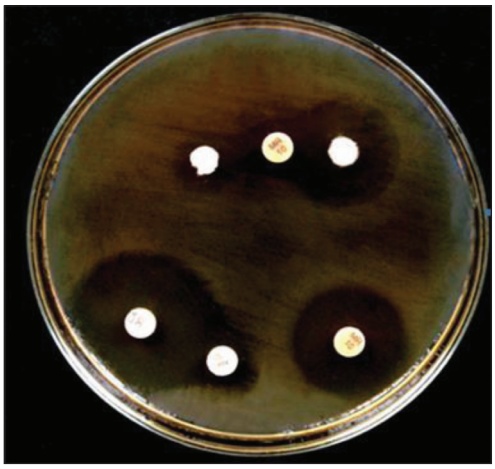

Figure 2: Detection of carbapenem resistance by double disk synergy test. Meropenem disks are flanked by plain disks of EDTA and Zinc sulphate. Molecular Class B MBLs producing positive isolates showing zone towards EDTA and Zn disks

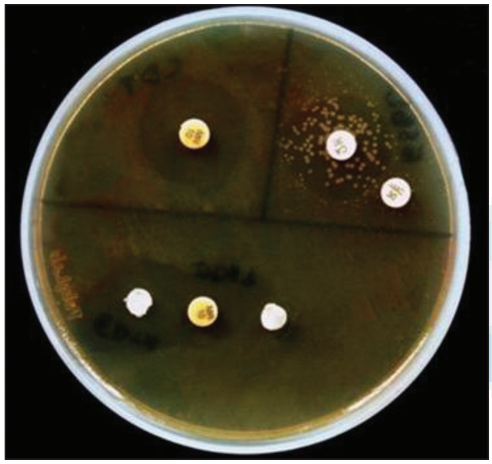

Figure 3: ESBL double disk synergy test with amoxyclav and ceftazidime showing heteroresistance.

identification test. Hence compared to all the phenotypic tests for MBL detection DDST is reliable and reproducible, with separate EDTA and Zn disks it is also enabling identification of ambler class B MBLs but freshly prepared EDTA gives good result. Heteroresistant isolates were observed in ESBL double disk synergy test using amoxyclav and ceftazidime as in [Figure 3] in *Acinetobacter* spp, but had clear zone for MBLs detection.

Low-level resistance to carbapenems as determined by MIC has been reported in several studies[12] which were detected resistant by disk diffusion method. All the 21 meropenem-resistant isolates were also resistant to most of the other antimicrobials tested one of the major concerns emerging from this study is the fact that six meropenem-resistant isolates were resistant to cefoperazone-sulbactam also. If carbapenemases spread widely there will be a situation where many nosocomial Gram-negative infections become untreatable so a simple phenotypic test in hospitals for differentiating MBLs is very critical for treating ICU patients. Further studies are underway to confirm the positive molecular class B MBLs producing strains and standardize this putative test.

Acknowledgement

We thank Dr.Indumathi VA, Gokula Metropolis Clinical Laboratories, Bangalore -560 054, for providing generous gifts of bacterial strains with plasmids.

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How to cite this article: John S, Balagurunathan R. Metallo beta lactamase producing Pseudomonas aeruginosa and Acinetobacter baumannii. Indian J Med Microbiol 2011;29:302-4.

Source of Support: Nil, **Conflict of Interest:** None declared.