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Research Article

PHENOTYPIC DETECTION OF METALLO BETA LACTAMASE IN GRAM NEGATIVE BACTERIAL ISOLATES

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ABSTRACT
Carbapenem Resistance due to the production of metallo-beta-lactamases (MBL) in Gram-negative organisms is an increasing international public health problem. Emergence of MBL producing organisms is alarming and reflects the excessive use of carbapenems. The problem of MBL producing strains was originally confined to *Pseudomonas* and *Acinetobacter*. However, carbapenem resistance has been observed in members of Enterobacteriaceae family due to spread of MBL genes. The present study was aimed at determining the prevalence of metallo-beta-lactamases (MBL) production in gram negative bacterial isolates obtained from various clinical isolates. A total of 1356 non-repeat isolates of gram negative bacterial isolates obtained from various clinical samples were processed by conventional methods. Out of these 1356 isolates, only those which were resistant to meropenem by the E-test method were included in this study. Among these resistant strains, MBL production was determined by the combined disk test (CDT). Out of 1356 non-repeat isolates of gram negative bacterial isolates,112 isolates showed resistance to meropenem by the E-test method. Out of which 74.11% of the isolates turned out to be MBL producers by the CDT method. In this study, 85% of *Pseudomonas* species, 75.44% of *Acinetobacter*, 66.67% of *Klebsiella*, 50% each of *E.coli* and *Citrobacter* species were MBL positive.The maximum number of MBL producers were reported from respiratory samples followed by plastic devices. The higher prevalence rate of MBL producing organisms in the present study focuses on the need for strict implementation of infection control practices and antibiotic restriction policies to avoid excessive use of carbapenems and other broad-spectrum antibiotics.

Keywords: *Carbapenems, Metallo-beta-lactamases (MBL), Gram negative bacteria, Meropenem, Combined disk test (CDT).*

INTRODUCTION
The introduction of carbapenems into clinical practice was of great help in the treatment of serious bacterial infections caused by β-lactam resistant bacteria (Varaiya *et al.*, 2008). Carbapenems are often used as antibiotics of last resort for treating infections due to multidrug-resistant gram-negative bacilli, because they are stable even in response to extended-spectrum and AmpC β-lactamases (Lee *et al.*, 2003). The emergence of acquired metallo- β-lactamases (MBLs) in Gram-negative bacilli is becoming a therapeutic challenge, as these enzymes usually possess a broad hydrolysis profile that includes all βlactam antibiotics with the exception of monobactams (Galani *et al.*, 2008). Unlike carbapenem resistance due to several other mechanisms, the resistance due to MBL and other carbapenemase production has a potential for rapid dissemination, as it is often plasmid mediated. (Walsh *et al.*, 2005) In recent years, MBL genes have spread from *P. aeruginosa*to members of the Enterobacteriaceae family (Peleg *et al.*, 2005).
In addition to their resistance to all β-lactams, the MBL producing strains are frequently resistant to aminoglycosides and fluoroquinolones. MBL producing isolates are also associated with a higher morbidity and mortality (Walsh *et al.*, 2005). Moreover the treatment alternatives are unavailable or expensive/ toxic with poor outcome (Marra *et al.*, 2006).
Based on molecular studies, carbapenemhydrolyzing enzymes are classified into four groups A, B, C and D. The MBLs belong to group B and are enzymes requiring divalent cations as cofactors for enzyme activity, being inhibited by the action of a metal ion chelator (Ambler *et al.*, 1980). Although PCR-based

genotyping remains as the golden standard for MBL detection and classification, its use is mainly restricted to research purposes (Khosravi *et al.*, 2012).

Several non-molecular techniques have been studied, all taking advantage of the enzyme's zinc dependence by using chelating agents, such as EDTA or 2 mercaptopropionic acid, to inhibit its activity. (Franklin *et al.*, 2006).There is a global increase in the prevalence of MBL-producing non-fermenting bacilli and Enterobacteriaceae (Walsh *et al.*, 2005; Galani *et al.*, 2008). Therefore this study was done to find the prevalence of Metallo beta lactamases among the gram negative bacteria isolated from various clinical samples.

MATERIALS AND METHODS

It was a prospective study conducted between October 2012-September 2013 at the department of microbiology of Dr B.R Ambedkar Medical College, Bangalore. A total of 1356 non-repeat isolates of gram negative bacteria were included in this study. The isolates were identified by conventional methods (Collee, 1999). Repeated isolates from the same patient were excluded. Antibiotic susceptibility testing was performed by the disc diffusion method using Mueller–Hinton agar (HIMEDIA) as per the CLSI 2012 guidelines. The MIC of meropenem was determined by the E test, according to the manufacturer's recommendations (BioMérieux, Marcy l'Etoile, France) and interpreted according to the CLSI 2012. Only meropenem resistant isolates were included in this study. These isolates were also resistant to other carbapenems and third generation cephalosporins. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as controls.

The meropenem resistant isolates were then subjected to the combined disk test (CDT) for the detection of MBL.

Combined disktest (CDT):

The MEROPENEM-EDTA combined disk test was performed as described by Yong *et al.*, 2002. Test organisms were inoculated on to plates with Mueller Hinton agar as recommended by the CLSI. Two 10 µgmeropenem disks (Becton Dickinson) were placed on the plate, and appropriate amounts of 10 µL of EDTA solution were added to one of them to obtain the desired concentration (750 µg). The inhibition zones of the meropenem and meropenem-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 meropenem and EDTA disc was ≥ 7 mm than the meropenem disc alone, it was considered as MBL positive.

RESULTS

Out of which 1356 non-repeat isolates of gram negative bacterial isolates, 112 isolates showed resistance to meropenem by the E-test method. 57 out of 112 isolates screened were Acinetobacter species, 40 were Pseudomonas species, 9 were Klebsiella , 4 were E.coli and 2 were Citrobacter species.

Among the resistant strains, 85% of *Pseudomonas* species, 75.44% of *Acinetobacter*, 66.67% of *Klebsiella*, 50% each of *E.coli* and *Citrobacter* species were MBL positive by the Combined disk diffusion test (Table 1).

Table 1: Distribution of MBL producing organisms

Organism isolated	Meropenem resistant(%)	MBL positive by CDT (%)
Pseudomonas	40 (35.71%)	34 (85%)
Acinetobacter	57 (50.9%)	43 (75.44%)
Klebsiella	9 (8.06%)	6 (66.67%)
E.coli	4 (3.57%)	2 (50%)
Citrobacter	2 (1.8%)	1 (50%)
	112	86 (74.11%)

The maximum number of MBL positive organisms were isolated from the respiratory samples which included sputum and endotracheal aspirates followed by plastic devices which included central and peripheral venous catheters and urinary catheters (Table 2 and Chart 1).

Table 2: Distribution of MBL producing bacterial isolates in various clinical samples :

	Respiratory samples	Pus	Urine	Blood	Genital samples	Plastic devices	Total
Acinetobacter	17	7	2	3	2	12	43
Pseudomonas	12	5	2	2	1	12	34
Klebsiella	4	1	1	0	0	0	6
E.coli	0	0	0	1	0	1	2
Citrobacter	0	1	0	0	0	0	1
Total	33	14	5	6	3	25	86

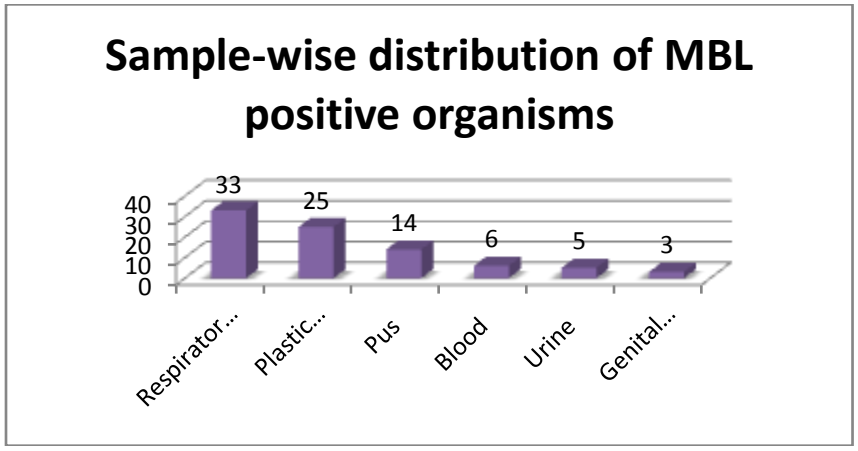

Chart 1: Sample-wise distribution of MBL positive organisms

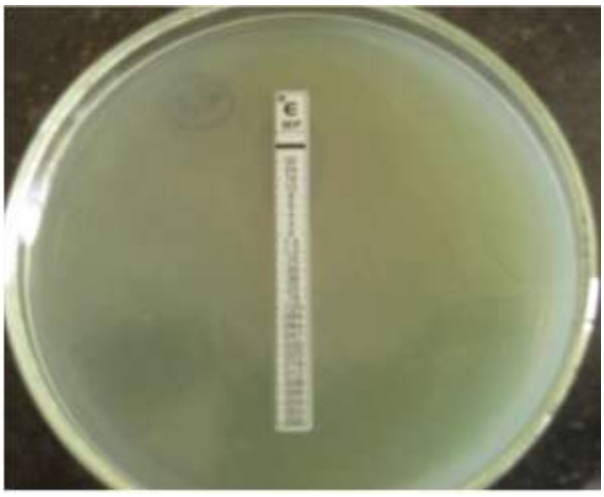

Figure 1: Meropenem E-test: Pseudomonas aeruginosa showing MIC ≥32 µg/ml

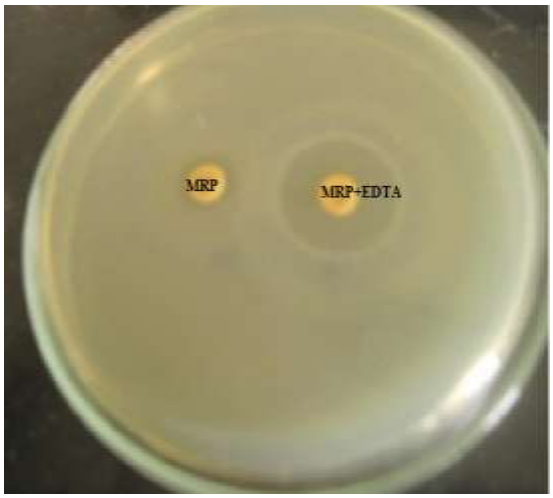

Figure 2 : Combined disk test (CDT) : Zone of inhibition of MRP+EDTA disc is ≥7 mm than that of MRP disc alone

DISCUSSION

Carbapenem Resistance due to the production of metallo-beta-lactamases (MBL) in Gram-negative organisms is an increasing international public health problem (Walsh *et al.*, 2005; Cornaglia *et al.*, 2007) Genes encoding for MBL were shown to be carried on large transferable plasmids or were associated with transposons, allowing horizontal transfer of these MBL genes among different bacterial genera and species (Pitout *et al.*, 2007).

Carbapenemhydrolyzing 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 our study, *Acinetobacter* species showed maximum resistance to meropenem (50.9%). Similar resistance rates have been reported by other studies (Manikal *et al.*, 2000; Noyal *et al.*, 2009). Even higher rates have been reported by Nazmul *et al.*, 2012 (92.5%). *Pseudomonas* species showed resistance of 35.71% which also correlates with studies done by Bhat *et al.*, 2013 followed by *Klebsiella* species which showed a resistance rate of 8.06%. This is in concordance with Hodiwala *et al.*, 2013 and Pandya *et al.*, 2011.

The maximum number of these resistant isolates was obtained from respiratory samples which included endotracheal aspirates and sputum followed by plastic devices (catheter tip), pus, blood and urine indicating that the use of indwelling medical devices plays an important role in the spread of infective agents. Other studies have also reported a higher rate of resistant isolates from respiratory samples (Jesudasan *et al.*, 2005; Kaleem *et al.*, 2010).

Few studies have reported that 'Imipenem-EDTA combined disk test' as the most sensitive method for detection of MBL production in gram negative bacilli (Franklin *et al.*, 2006 and Pandya *et al.*, 2011). Hence we followed the combined disk method using meropenem- EDTA for the detection of MBL in the gram negative organisms.74.11% of the meropenem 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.

Pseudomonas species showed the highest MBL production rate accounting to 85%, followed by Acinetobacter species (75.44%) and Klebsiella (66.67%). Pandya *et al.*, 2011 also observed a similar pattern of MBL resistance rate in their study on Gram negative bacterial isolates.

83.8% of *P. aeruginosa*were MBL positive in study done by Gupta *et al.*, 2013 and Hemalatha *et al.*, 2005 also reported 87.5 % MBL rate in *Pseudomonas.*

Acinetobacter species exhibited MBL production rate of 75.44% which correlates with study done by Tellis *et al.*, 2013. Higher rates (84% and 96%) have been observed by other studies in Pakistan (Kaleem *et al.*, 2010; Irfan *et al.*, 2008 respectively). Whereas studies done by Noyal *et al.*, 2009 and Anil *et al.*, 2011 in India have reported lower prevalence of MBL in *Acinetobacter* species (6.5% and 21% respectively). 66.67% of the Klebsiella isolates were MBL positive. These results were comparable to a study done by Jain *et al.*, 2012.

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.

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