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RESEARCH ARTICLE

Carbapenem susceptible metallo beta lactamase producing gram negative bacilli: beware of wolf in sheep dress.

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Abstract

Context: MBL producing gram negative bacilli are associated with serious infections and failure of therapy. Many isolates carrying the MBL gene have been found susceptible to imipenem. These so called "hidden MBLs" create a significant problem with respect to their detection. Such organisms may pose a significant risk, particularly due to their role in unnoticed spread within institution and their ability to participate in horizontal transfer with other pathogen related organisms

Aims: This study was undertaken to determine the prevalence, risk factors and treatment outcome of carbapenem susceptible metallo beta lactamase producing gram negative bacteria.

Methods and Material: Gram negative bacteria resistant to ceftazidime, ticarcillin and ceftazidime clavulanate / ticarcillin clavulanate but susceptible to imipenem were included in the study. Phenotypic detection of metallo beta lactamases involved combined disk test (CDT), double disk synergy test (DDST) and susceptibility to aztreonem. MIC was determined by agar dilution method (for imipenem and imipenem-EDTA) as well as Etest.

Results: Out of 119 isolates included in the study, a total of 30 (25.21%) isolates were found to be MBL producers. A fourfold reduction in MIC by imipenem-EDTA combination compared to imipenem was also found to be a sensitive indicator for the detection of MBL carrying isolates. Prolonged hospitalization and indwelling urinary catheterization were the possible risk factors.

Conclusion: This study suggests the use of a simple and highly sensitive method for the detection of MBL production in carbapenem susceptible isolates of gram negative bacteria in resource poor setting.

Key messages: Carbapenem susceptible metallo beta lactamase producing gram negative bacilli are a threat to infection control programs. Appropriate and sensitive phenotypic methods are available for their detection.

INTRODUCTION:

Carbapenems are used as antibiotics of last resort in the management of infections caused by gram negative bacteria due to their broad spectrum of activity and stability to most β- lactamases. Resistance against this class of

drugs may develop due to the production of metallo beta lactamases (Nordmann and Poirel, 2002). Metallo- β-lactamases (MBL) belong to the molecular class B of the Ambler & in group 3 of the Bush Jacoby Medeiros functional classification **(**Hall and Barlow, 2005**;** Bush et al., 1995). The MBLs are characterized by their ability to hydrolyze penicillins, cephalosporins and carbapenems, but lack the ability to hydrolyze aztreonam and are susceptible to inhibition by metal ion chelators such as EDTA (Nordmann and Poirel, 2002; Walsh et al., 2005). They can be either chromosomally encoded or acquired (transferable MBLs). These transferable MBLs are often encoded by mobile genetic elements such as cassettes inserted in to integrons. When these integrons become associated with plasmids or transposons, transfer between bacteria is facilitated enabling widespread dissemination (Walsh et al., 2005; Pitout et al., 2005; Franklinet al., 2006).

MBL producing gram negative bacilli are associated with serious infections and failure of therapy poses serious therapeutic challenge. Many isolates carrying the MBL gene have been found susceptible to imipenem. These so called "hidden MBLs" create a significant problem with respect to their detection. As a result of being difficult to detect, such organisms pose a significant risk, particularly due to their role in unnoticed spread within institution and their ability to participate in horizontal transfer with other pathogen related organisms. Also they can be threat to infection control efforts and may result in poor clinical outcome of patients with serious infection (Walsh et al., 2005; Franklin et al., 2006).

This study was undertaken to determine the prevalence of metallo-beta-lactamases in carbapenem susceptible isolates of gram negative bacteria. Attempts were made to find out risk factors, treatment outcome and possible presence of metallo-beta-lactamases producing organisms in the community.

Material and Methods

This prospective study was conducted in tertiary care hospital, over a period of 1 year. Gram negative bacilli isolated from various clinical samples of patients admitted or attending OPD units were identified using conventional techniques. Antimicrobial susceptibility was performed by Kirby-Bauer disk diffusion method according to CLSI guidelines (CLSI, 2010). Tigecycline (15µg) procured from Becton, Dickinson and company 7 Loveton Circle sparks, Maryland 21152 USA, was also evaluated for its antimicrobial activity against the all MBL positive organisms except Pseudomonas. Escherichia coli ATCC 25922 and Pseudomonas ATCC 27853 were used as negative control. An inhouse MBL positive isolate served as a positive control.

Inclusion criteria: Gram negative bacilli susceptible to imipenem (IPM) but resistant to ceftazidime, ticarcillin and ceftazidime clavulanate / ticarcillin clavulanate were taken as an initial threshold for pursuing metallo beta lactamase detection (Franklin et al, 2006).

Exclusion criteria: Gram negative bacilli susceptible to ceftazidime, ticarcillin and ceftazidime clavulanate / ticarcillin clavulanate and polymicrobial infections were excluded from the study.

Preparation of EDTA disk: The imipenem EDTA disks were prepared by adding 10µl of 0.1M (292µg) EDTA solution to imipenem disk. The disks were dried immediately in an incubator and stored at -20ºC till used (Yan et al, 2004).

Phenotypic detection of MBLs: A 1 in 10 dilution of 0.5 Mc Farland standard of organism suspension was inoculated on the Mueller Hinton agar (Hi-Media Laboratories Pvt. Limited; Mumbai India). Phenotypic detection of MBLs comprised of 3 components (Franklin et al, 2006):(Fig 1)

a. **Combined disk test (CDT):** In CDT, 2 imipenem disks (10µg), one containing 10µl of 0.1M (292 µg) anhydrous EDTA was placed 25mm apart (center to center). An increase in zone diameter of >4mm around IPM-EDTA (imipenem-EDTA) disk compared to IPM (imipenem) disk alone was considered positive for MBL.
b. **Double Disk Synergy Test (DDST):** In DDST, an IPM (10µg) disk was placed 20mm (center to center) from a blank disk containing 10µl of 0.1M (292µg) EDTA. Enhancement of zone of inhibition in the area between the 2 disks was considered positive for MBL.
c. **Aztreonam Disk:** The final component was to assess the susceptibility to aztreonam (30µg) disk and to determine the utility of this component in phenotypic detection of MBL.

Determination of MIC:

MIC was determined by agar dilution method as well as by Etest method. MIC by agar dilution method was determined for both imipenem as well as imipenem-EDTA combination as was proposed by Hemalatha et al (Hemalatha et al., 2005). MIC of imipenem and imipenem-EDTA combination by E-test method was read where the inhibition ellipses intersected the strip (Yan et al, 2004).

Detailed information of the patients and isolates was recorded. The medical records of the patients were reviewed for age, sex, days of hospitalization, underlying illness, severity of illness as calculated by simplified acute physiology score (SAPS), presence of malignant disease and related treatment, any surgical procedure, site of infection, source from which isolate was recovered, presence of devices (urinary catheter, CVC), previous antibiotic use etc (Hirakata et al.,2003; Gall et al., 1993). Patients were divided into 2 categories of clinical significance:

a. **Infected:** If the patient showed both an acute systemic inflammatory response (e.g. fever) and local signs and symptoms of infection (e.g. purulent sputum, pyuria)
b. **Colonized or unknown:** If the patient displayed no signs and symptoms, even if the patient had a systemic inflammatory response or if sufficient information was not available from the patient

Outcome: Outcome of the patient was recorded and classified as

a) **Recovery** (from signs and symptoms related to infection)

b) **Death/Failure** (no resolution or worsening of signs and symptoms)

c) **Indeterminate** (inability to assess due to lack of follow-up information)

Statistical Analysis: The data was expressed as mean ± SD and the percentage. The inter-group comparison and risk estimation was performed using chi-square test and odds ratio. The statistical package of Java stat calculators was used.

Ethical clearance: The study was approved by the Institutes' ethical clearance committee.

Results:

Of the 1560 non duplicate gram negative bacilli isolated during the study period, 119 isolates were resistant to ceftazidime/ticarcillin and ceftazidime-clavulanate/ ticarcillin-clavulanate thus fulfilling the criteria to be included in the study. Of these, a total of 30 (25.21%) isolates were found to be MBL producers and 89 (74.79%) were MBL negative. When evaluated by different phenotypic methods, 22 (73.3%) of the 30 MBL carrying isolates were positive in both combined disk test (CDT) and double disk synergy test (DDST). (Fig 1) Eight (26.7%) isolates were positive by combined disk test only. All the MBL carrying isolates demonstrated 4- 10 fold reduction in MIC in the presence of imipenem- EDTA combination by agar dilution test. (Table 1) Etest was performed for 10 representative isolates. These isolates had an MIC of 4 or less than 4 for imipenem and MIC of less than 1 for imipenem- EDTA combination. (Fig 2).

For isolates confirmed to be MBL producers, the mean age was 51 years (range 1month -82 years). The predominant source of MBL producing isolates were wound swabs and pus (n=17; 56.7%), followed by urine (n=7; 23.3%), blood (n=3; 10%) and body fluids (n=3; 10%). Isolation of MBL isolate from urine was found to be statistically significant (P= 0.023). Pseudomonas was most predominant organism isolated (n=12; 40%). Other organisms isolated were *Escherichia coli* 9 (30%), *Klebsiella* spp. 5 (16.7%), *Acinetobacter* spp 2 (6.7%), *Proteus* spp 1 (3.3%), and *Citrobacter* spp 1(3.3%). Out of 11 isolates screened for MBL detection from the outpatient department, one (3.3%) was positive for MBL production. Majority (80%) of patients had a SAPS score of < forty. Of the MBL positive, 5 (16.7%) were isolated from patients with burns, 7 (23.3%) from patients with trauma, 4 (13.3%) from patients with sepsis, 3 (10%) from patients with diabetes, 6 (20%) from patients with malignancy, 5 (16.7%) from patients with diagnosis other than the ones already stated.

Table 2 shows the association of risk factors for acquisition of MBLs. Fifteen (50%) patients with MBL producing isolates had undergone surgery while 6 (20%) patients had malignancy and 1 (3.3%) patient was on antineoplastic therapy (P > 0.05). Twenty-six (86.7%) patients had indwelling urinary catheter and the difrence from non producers was found to be statistically significant (P= 0.009). The organisms isolated belonged to different genera and there was no predominance of any genera. The recent use of β-lactam antibiotics and fluoroquinolones was more often noted in patients from whom MBL positive strains were isolated when compared to patients with non MBL producing strains (β-lactam antibiotics n=17, 56.7% in MBL producers Vs n= 43, 48.3% in non MBL producers; fluoroquinolones n= 12, 40% in MBL producers Vs n= 30, 33.3% in non MBL producers). However the difference in the use of antibiotics was not statistically significant (P> 0.05). The interval between hospital admission and isolation of the pathogen was longer among patients infected with MBL producers (mean 20.6 days, S.D ± 14.8) when compared to patients from whom non MBL producers were isolated (10.3 days, S.D ± 9.1). The difference was found to be statistically significant (P= 0.000).

The antimicrobial susceptibility pattern of MBL producers and non MBL producers did not vary much except that a significantly higher proportion of non MBL producing isolates were susceptible to piperacillin-tazobactam (P= 0.003). Sensitivity of MBL producers to aztreonam was seen in 11 (36.7%) isolates. The rest of the isolates (n=19) were resistant to aztreonam. A new drug tigecycline was evaluated for its activity against the MBL carrying *E. coli, Klebsiella, Acinetobacter* and *Citrobacte*r. It was found that all these isolates (100%) were sensitive to tigecycline.

Of the total 30 MBL producers, 13 organisms were isolated from patients with infection. MBL positive isolates produced more infection than MBL negative isolates (43.3% vs. 38.2% respectively). The mean days of hospitalization was higher in patients from whom MBL producing strains were isolated (n= 36 days, S.D ± 24) when compared to patients from whom non MBL producing strains were isolated (P= 0.002). Of the total patients with MBL positive isolates, 23(76.6%) recovered while 5 (16.7%) of the patients died (failure). Of the 5 patients who died, infection was thought to be cause of death in 2 patients (6.7% of all patients from whom MBL producing strains were isolated). These patients were treated with third generation cephalosporins. The outcome of 2 (6.67%) patients with MBL positive isolates could not be determined.

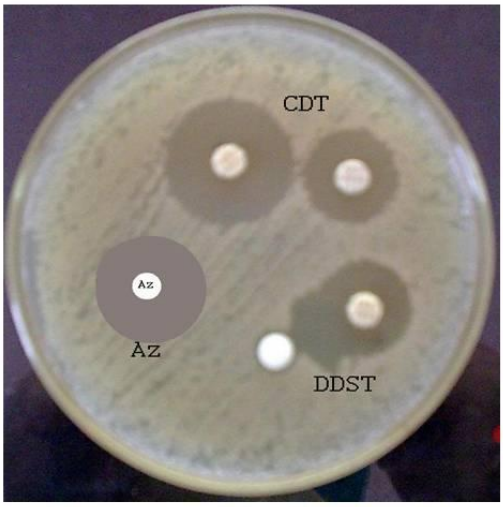

Fig I:: Phenotypic appearance of MBL producing isolates

Table 1: MIC of imipenem and imipenem+EDTA combination for MBL positive strains

Method	Agar dilution		E-test	
S. No.	Imipenem	Imipenem+ EDTA	Imipenem	Imipenem +EDTA
1	8	0.12	<4	<1
2	4	0.12	<4	<1
3	8	0.03	4	<1
4	8	0.06	4	<1
5	2	0.03		
6	8	0.015		
7	2	0.12		
8	4	0.12		
9	8	0.007		
10	2	0.03		
11	4	0.06		
12	2	0.03		
13	8	0.03	<4	<1
14	4	0.06		
15	2	0.12	<4	<1
16	2	0.12		
17	4	0.12		
18	4	0.015		
19	4	0.06		
20	4	0.06		
21	4	0.12	4	<1
22	2	0.03	4	<1
23	2	0.06	4	<1
24	4	0.12		
25	4	0.12		
26	2	0.06		
27	0.5	0.03		
28	2	0.06		
29	1	0.03	4	<1
30	4	0.12		

Table 2: Association of risk factors with MBL production

Risk factor	MBL positive		MBL negative		p value
	n (30)	%	n (89)	%	
1. Malignant disease					0.389 (NS)
Yes	6	20	12	13.5	
No	24	80	77	86.5	
2. Antineoplastic therapy					0.621 (NS)
Yes	1	3.3	5	5.6	
No	29	96.7	84	94.4	
3. Corticosteroid therapy					0.833 (NS)
Yes	2	6.7	5	5.6	
No	28	93.3	84	94.4	
4. Indwelling urinary catheter					0.009 (Sig.)
Yes	26	86.7	54	60.7	
No	4	13.3	35	39.3	
5. Surgery					0.557 (NS)
Yes	15	50	39	43.8	
No	15	50	50	56.2	
6. β- lactam intake					0.429 (NS)
Yes	17	56.7	43	48.3	
No	13	43.3	46	51.7	
7. Fluoroquinolone intake					0.533 (NS)
Yes	12	40	30	33.7	
No	18	60	59	66.3	

Discussion:

The frequency of antibiotic resistance in gram negative bacteria is worrisome since there are few, if any antibiotics in development, possessing suitable activity against these multi drug resistant organisms particularly, Pseudomonas & Acinetobacter spp. The increase in resistance is further enhanced by the acquisition of metallo β-lactamase genes (Queenan and Bush, 2007).

It has been seen that many of the isolates despite carrying the MBL genes are sensitive to imipenem. Such isolates are difficult to detect and therefore pose significant risk particularly due to their role in unnoticed spread within the institution and their ability to participate in horizontal MBL gene transfer with other pathogenic hospital related organisms (Franklin et al, 2006; Rossolini, 2005). Current knowledge strongly suggest that MBL producing organisms should be considered biologically resistant to carbapenem (regardless of the results of susceptibility testing) and these drugs should not be used to treat such infections. With the worldwide increase in occurrence and dissemination of metallo β-lactamases and increasing reports of carbapenem susceptible MBL producing isolates, early detection should be of paramount clinical importance. This study was undertaken to evaluate the presence of such carbapenem susceptible metallo β-lactamase producing gram negative bacilli in the laboratory.

In the present study, a lighter inoculum (1 in 10 dilution of 0.5 Mc Farland standard) and a lower concentration of EDTA (0.1M) were used. By this method, 30 (25.2%) isolates tested positive for MBL production. Of these, only 22 isolates were found positive in both combined disk test and DDST while 8 were positive in combined disk test only. This showed that combined disk test was more sensitive than DDST for the detection of metallo β-lactamases. Franklin et al reported a sensitivity of 100% and specificity of 98% by CDT whereas DDST had a sensitivity of 79% and specificity of 98% for MBL detection. Moreover, combined disk test was the preferred method because of its objective interpretation. The authors also suggested that modifications in methodology employed, inoculum size & low concentration of EDTA were helpful in maximizing the detection of such isolates (Franklin et al, 2006).

Aztreonam susceptibility is a common feature of MBL producing organisms. However in present study it was found that 11 (36.7%) of the isolates were sensitive while 19 (63.3%) were resistant. The resistance to aztreonam could be best explained by the presence of other mechanisms of resistance, most importantly AmpC β-lactamase or combination of permeability defects and efflux mechanisms (Franklin et al, 2006; Peleg et al, 2005;

Cornaglia et al, 2000). Varying susceptibility to aztreonam has been found in different studies (Hirakata et al, 2003; Peleg et al, 2005; Yan et al, 2001; Lee et al, 2009). The clinical utility of aztreonam in phenotypic MBL detection in our study was limited possibly due to other co-existing resistance mechanisms.

Overall MBL carrying isolates had an MIC for imipenem in the susceptible range (0.06-16 for Pseudomonas & 0.06-4 for Enterobacteriaceae). This variable carbapenem susceptibility has been described previously in several other studies and can be explained by number of factors (Cornaglia et al, 2000; Lee et al, 2009; Senda, 1996a). Firstly phenotypic expression of resistance is more likely when other resistance mechanisms such as co-presence of up-regulated efflux pumps and/or membrane impermeability are present e.g in P. aeruginosa isolates. Other explanations include suppressed MBL gene expression by secondary regulatory systems, leading to a silent or cryptic bla_{IMP} gene, and varied carbapenem hydrolysis depending on the MBL gene dosage effect, which relates to the plasmid copy number (Peleg et al, 2005).

A fourfold reduction in MIC was observed with imipenem-EDTA combination when compared to imipenem alone (Table 1). The commercially available E-test (Fig 2) did not prove to be sensitive for MBL detection as the MIC of most of the isolates was 4 or less than 4 for imipenem and less than one for IMP-EDTA and the minimal concentration of imipenem with and without on the E-test MBL strip were 1 and 4μg/ml respectively. It was validated that antimicrobial susceptibility and MIC obtained by conventional testing is neither specific nor a sensitive indicator for detection of MBL carrying organisms. Therefore a phenotypic method comprising of combined disk test (CDT) and double disk synergy test (DDST) is recommended for the detection of MBL producing isolates (Franklin et al, 2006). In our study, a four fold reduction in MIC by imipenem-EDTA combination compared to imipenem was also found to be a sensitive indicator for the detection of MBL carrying isolates. However, the method requires preparation of several fold dilution of the antibiotic solution which is cumbersome.

The isolation of MBL positive strains in urine had a statistically significant association as has been seen in other studies as well. Hirakata et al attributed the similar observation in their study to the use of indwelling urinary catheter (Hirakata et al, 2003). Senda et al reasoned that bacteria surviving in urine must acquire high level resistance to broad spectrum β-lactams excreted in urine or bile without being dissolved or modified. For this purpose, metallo β-lactamase resistance is of great benefit to these bacteria (Senda et al, 1996b).

Among the MBL positive isolates, the most commonly isolated organism was Pseudomonas (12 isolates) followed by Escherichia coli (9 isolates), Klebsiella (5 isolates), Acinetobacter spp (2 isolates), Proteus (1 isolate) and Citrobacter (1 isolate). The presence of MBL determinant in several genera reflects the ease with which the horizontal transfer of MBL genes occur. This finding should alert us on the further dissemination of MBL determinants among various gram negative bacilli (Peleg et al, 2005; Senda et al, 1996b).

Our studies indicate that age and gender distribution of patients infected or colonized with MBL positive strains was not significantly different from non MBL producers. Majority of the MBL producing organisms were isolated from inpatients suggesting that metallo β-lactamase producing isolates are mostly nosocomial in origin. One MBL producing strain was isolated from an out-patient department. The patient had a history of prior hospitalization and was presently suffering from recurrent urinary tract infection. The history of prior hospitalization in this patient excludes the possibility of community origin of MBLs.

A statistically significant association was found between long term hospitalization and the isolation of the pathogen. Hirakata et al also found prolonged hospitalization as a risk factor for the acquisition of metallo β-lactamase (Hirakata et al, 2003). To assess the risk factors associated with the acquisition of metallo β- lactamases (Table 2), our results indicated a statistically significant association between MBL producing organisms and use of indwelling catheters. A similar observation was made by Hirakata et al. Urinary catheterization has also been reported as a risk factor for the MBL producing Pseudomonas aeruginosa by Zvascki et al (Zvascki et al, 2006). In our study only 3 (10%) of the 30 patients had a history of carbapenem intake. This observation suggests that selective pressure from carbapenems is not required for the isolation of MBL producers. Our data is consistent with that obtained by Hirakata et al and Yan et al (Hirakata et al, 2003; Yan et al, 2001).

MBL producing isolates were resistant to several antimicrobial classes. Tigecycline was found to have considerable antimicrobial activity against MBL producing Enterobacteria and Acinetobacter species. Therefore tigecycline can be used as a reserve drug for the treatment of such multi-drug resistant, MBL producing organisms other than Pseudomonas.

The total length of hospital stay was prolonged in patients colonized or infected with MBL producing organisms (mean 36 days; range 0-103 days) compared to the patients with non MBL producing strains (mean 23.3 days; range 0-80 days). In our study, 76.6% of the patients infected or colonized with MBL producers recovered while 84.3% of the patients with non MBL producers recovered. The proportion of patients who died after infection was higher in patients with MBL producers than among the non MBL producers. The prolonged duration of hospital

stay and increased case fatality rate in MBL producers suggest that the clinical condition of MBL positive patients was more severe than that of the non MBL producers.

MBL producing isolates are a cause of remarkable clinical concern as they are associated with resistance to many classes of potent antimicrobial agents including β-lactams, aminoglycosides, fluoroquinolones and carbapenems (Walsh et al, 2005; Yan et al, 2001). Carbapenem susceptible isolates have been found to produce MBL and these have been referred to as wolf in sheep's dress. Such organisms often carry hidden MBL genes whereby the microbiologist and clinician remain unaware of their presence within an institution. Such a scenario creates a potential for untoward clinical and infection control consequences. Perhaps most disturbing is the dearth of new drug options. This emphasizes the need for continuous surveillance of these emerging resistance determinants. This study suggests the use of a simple and highly sensitive method for the detection of MBL production in carbapenem susceptible isolates of gram negative bacteria particularly in resource poor setting where molecular methods are not available.

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