JOURNAL OF CLINICAL AND DIAGNOSTIC RESEARCH

How to cite this article:
RENU GOYAL, RAJEEV THAKUR, SMITA SARMA. THE EXISTENCE OF METALLO BETA LACTAMASES IN CARBAPENEM SUSCEPTIBLE GRAM NEGATIVE BACLLI: A CAUSE FOR CONCERN. Journal of Clinical and Diagnostic Research [serial online] 2010 August [cited: 2010 August 31]; 4:2679-2684.

Available from
http://www.jcdr.net/back_issues.asp?issn=0973-709x&year=2010 &month= August &volume=4&issue=4&page=2679-2684 &id=1126

ORIGINAL ARTICLE

The Existence Of Metallo Beta Lactamases In Carbapenem Susceptible Gram Negative Bacilli: A Cause For Concern

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ABSTRACT

Background: Metallo-ß-lactamases (MBLs) have been increasingly recognized among imipenem resistant isolates, but they also appear to exist in imipenem susceptible isolates. These undetected hidden MBLs in sensitive isolates can spread unnoticed in hospitals if such isolates are reported susceptible to carbepenem without screening for the presence of MBLs. The laboratory detection of such isolates is crucial as they pose a serious therapeutic challenge.

Aims: The aim of our study was to detect MBLs in both the imipenem resistant and sensitive isolates by using combination of available phenotypic methods.

Settings and design: This was a hospital based prospective study which was carried out in a tertiary neuropsychiatric centre from April 2008 to April 2009.

Methods and Material: A total of 130 gram negative isolates (50 imipenem sensitive, 30 imipenem intermediate and 50 imipenem resistant by the Kirby Bauer disc diffusion method) were tested for the presence of MBLs by the double disk synergy test (imipenem, EDTA 750µg/ml), the combined disk test (imipenem, imipenem + EDTA 750µg/ml) and the MBL E test strip.

Statistical analysis: Descriptive statistics was used and the percentages of MBLs carrying imipenem resistant and sensitive isolates were calculated.

Results: MBLs were detected in 11 (20%) imipenem sensitive isolates. Out of these 11 imipenem sensitive MBL producing strains, 8 were from the imipenem sensitive category and 3 were from the intermediate category. These 3 isolates in the intermediate category had MIC for imipenem in the sensitive range (MIC ≤4 ug/ml) by E test method. All the MBL carrying imipenem sensitive isolates had zone diameters in between 16 to 22 mm by the Kirby Baeur disc diffusion method. Among the imipenem resistant isolates, 38 isolates were MBL producers (32 from the imipenem resistant category and 6 from the intermediate category).

Conclusions: This study reports the existence of MBLs in carbapenem susceptible organisms and proposes that gram negative bacterial isolates having an imipenem zone diameter ≤22mm by the disk diffusion method should be routinely screened for presence of MBLs.

Key-words: Metallo-ß-lactamases (MBLs), antibiotic resistance, imipenem, Double disk synergy test (DDST)

KEY MESSAGES:

- Metallo-ß-lactamases (MBLs) are not only restricted to the carbapenem resistant strains, but are also present in carbapenem susceptible organisms.
- These carbapenem susceptible organisms with hidden MBL genes can spread unnoticed in hospitals if such isolates are reported as sensitive without screening for the presence of MBLs.
- The laboratory detection of MBL carrying organisms is of significant clinical importance to stop their uncontrolled spread and the emergence of antimicrobial resistance.

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INTRODUCTION

Till the emergence of the carbapenemases, carbapenems were the drugs of choice for penicillin resistant or cephalosporin resistant gram negative bacilli; as these were stable to hydrolysis by most β-lactamases (extended-spectrum and AmpC ß-lactamases).[1],[2] These carbapenemases are most often metallo-ß-lactamases (MBLs) which are capable of hydrolyzing not only carbapenems, but also all ß-lactam antibiotics except aztreonam.[1],[2],[3] MBLs are resistant to classical β-lactamase inhibitors, but are susceptible to EDTA and thiol-based compounds.[4][5],[6],[7] IMP-1 MBL was first reported from Japan, from *Serratia marcescens* and *Pseudomonas aeruginosa*, after which several variants of MBLs like IMP 1 (IMP-2 to 9), VIM, SPM and GIM have been detected and characterized worldwide. [1],[2],[6],[10]Although scanty data is available on the overall prevalence of these enzymes among clinical isolates, a particular concern is that acquired MBL genes are located on integron structures that reside on mobile genetic elements such as plasmids or transposons and can widely disseminate in hospitals.[8]

These MBLs, as thought earlier, are just not restricted to the carbapenem resistant strains, but some recent reports have argued about their presence in carbapenem susceptible organisms also.[11],[12] As seen with extended spectrum beta lactamases (ESBLs) and AmpC type lactamases with cephalosporins, MBL carrying organisms can appear to be susceptible to carbapenems by current clinical and laboratory standard guidelines.[13] These carbapenem susceptible organisms with hidden MBL genes can spread unnoticed in hospitals if such isolates are reported as sensitive without screening for the presence of MBLs. The treatment of these organisms pose a serious therapeutic challenge as these strains are most often resistant to multiple drugs and can spread unnoticed in hospitals along with other hospital related organisms.[1],[2] The laboratory detection of carbapenem susceptible MBL carrying organisms is of significant clinical importance in order to stop their uncontrolled spread.

The present study was undertaken to detect metallo- β- lactamases in carbapenem resistant and susceptible isolates by the double-disk synergy test (DDST), combined-disk test (CDT) and an MBL Etest strip..

METHODS AND MATERIALS

***Bacterial strains*:** A total of 50 consecutive, non repeat, IMP-resistant (Zone diameter ≤13), 30 intermediate (Zone diameter 14-15mm) and 50 IMP sensitive (zone diameter ≥16) gram-negative bacterial isolates obtained from various clinical specimens were included in the study.[13] All the isolates were characterized up to the species level by using standard microbiological techniques.[14] A list of the bacterial strains which were tested and their source of isolation is shown in Table 1.

Antimicrobial susceptibility testing was done for all the bacterial isolates by using commercially available disks (Himedia, Mumbai, India) in accordance with the CLSI guidelines.[13] The antibiotics which were tested were piperacillin 100 µg (PIP), ceftazidime 30 µg (CAZ), imipenem 10 µg (IPM), ciprofloxacin 5µg (CIP), gentamicin 10 µg (GEN) amikacin 30 µg (AK) and aztreonam 30 µg (ATM). The quality control strains which were used were *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

The Phenotypic MBL detection method: All the isolates were screened for the presence of MBLs by the double-disk synergy test (DDST), the combined-disk test (CDT), and the MBL E test strip (AB BioDisk Company, Sweden).

The DDST and the Combined Disk Test: A 0.5 M EDTA solution was prepared by dissolving 186.1 gm of EDTA. $2H_2O$ in 1000 ml of distilled water and its pH was adjusted to 8.0 by using NaOH. This mixture was sterilized by autoclaving.[7]

For the **combined disk test,** two 10 µg IMP discs were placed on the surface of an agar plate and 5µl EDTA solution was added to one of them to obtain a concentration of 750 µg. The inhibition zones of IMP and IMP-EDTA were compared after 16-18 hours of incubation in air at 35^0C. An increase in zone size to ≥7 mm was taken as positive.[7],[12]

For the **disk synergy test,** an IMP disk was placed near a blank filter paper disk at a centre to centre distance of 10 to 25 mm. 5 µl of 0.5 M EDTA was applied to the blank disk (750 µg). After incubation for 16-18 hours, the presence of an enlarged zone of inhibition was interpreted as EDTA synergy test positive.[5] For the detection of MBLs in IMP susceptible isolates, IMP and a blank disk were kept at a distance of 25 mm.

***The MBL Etest procedure*:** MBL E test strips with IMP (4 to 256 µg/ml) and IMPE (1 to 64 µg/ml) were applied on Mueller-Hinton agar and were incubated for 16 to 20 hrs at 35°C. A reduction of IMP MIC ≥3 twofold dilutions in the presence of EDTA was interpreted as being suggestive of metallo-ß-lactamase production. Equally, the presence of a "phantom" zone between the two gradient sections or deformation of the IP ellipses was also indicative of the presence of metallo-ß-lactamases.[15]

The MBL E test strip was also used to detect MICs for IMP simultaneously with MBL ase detection. The MICs were interpreted as resistant, intermediate and sensitive as per the CLSI guidelines (Resistant: MIC≥ 16 ug/ml, sensitive: MIC 4< ug/ml).[13]

Ethics: All the procedures were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975 that was revised in 2000.

Statistical analysis:

Descriptive statistics was used and the percentages of MBLs carrying imipenem resistant and sensitive isolates were calculated.

RESULTS

All the isolates which were included in the study were multi drug resistant, with resistance to 4 or more drugs as shown in Table 2.

Table 3 depicts the MBL detection in different gram negative bacteria. Of the 50 IMP resistant isolates (MIC≥16 ug/ml), 32 isolates were detected as MBL producers by all three methods. Individually, 42 isolates were found to be positive by DDST, 32 by CDT and 32 by the MBL E test strip. Of the 42 DDST positive isolates, 32 isolates gave increase in the zone diameter of ≥7mm (CDT positive), while 4 isolates gave a zone diameter between 4 - 6 mm and 6 gave less than 4 mm.

Among 50 IMP sensitive isolates (MIC ≤4 ug/ml), 16 isolates had zone diameters varying from 16-20 mm (group A) and 14 isolates had zone diameters varying from 21-22 mm (group B), while 20 isolates had zone diameters ≥ 23 mm (group C). A total of 6 isolates from Group A, 2 isolates from Group B and none from Group C were detected as MBL producers by all the three methods. However, 8 isolates from Group A, 4 isolates from Group B and none from Group C were detected as MBL producers by the double disk synergy test. All the isolates which were positive for MBL by CDT were also positive by the MBL E test strip.

Of the 30 IMP intermediate isolates, 13 isolates were found to be MBL producers by the combined use of all the three methods. 15 isolates were found to be MBL producers by the DDST method. In the MBL E test strip method, the MICs of 6 isolates were found to be in the sensitive range, 16 were in the intermediate range and 10 were in the resistant range. Of these, 3 from the sensitive category, 4 from the intermediate category and 6 from the resistant category were found to be MBL producers.

Thus, a total of 11 imipenem sensitive isolates (8 from the sensitive and 3 from the intermediate categories) and 38 imipenem resistant isolates (32 from the resistant and 6 from the intermediate categories) were found to be MBL positive by all the three methods.

[Table/Fig 1]: Bacterial isolates and their source of isolation.

Bacterial genera	IMP resistant				IMP intermediate				IMP sensitive			
	Acineto	Pseud	E.coli	Kleb	Acineto	Pseud	E.coli	Kleb	Acineto	Pseud	E.coli	Kleb
RTI	3	5	1	1	5	5	1	1	7	5	2	1
Blood	5	4	-	-	1	-	-	-	-	-	-	-
Wound swabs	4	3	1	1	2	1	-	-	1	2	-	-
Body fluids	7	4	-	-	-	1	-	-	4	3	1	3
Urine	4	6	-	1	7	3	1	2	8	6	3	4
Total	23	22	2	3	15	10	2	3	20	16	6	8

RTI: Respiratory tract infections, Acineto: *Acinetobacter baumanii*, Pseud: *Pseudomonas aeruginosa*, E. coli: Escherichia coli, kleb: *Klebsiella pneumoniea*

[Table/Fig 2]: Drug resistant profile of all the isolates.

Drug resistant profile	*Acinetobacter*			*Pseudomonas*			*E. coli*			*Klebsiella*		
	R	I	S	R	I	S	R	I	S	R	I	S
CAZ, PIP, CIP, ATM, AK, GEN	6	5	7	5	2	3	1	-	1	-	-	-
CAZ, CIP, ATM, AK, GEN	1	1	-	1	-	1	-	-	-	1	1	2
CAZ, PIP, CIP, AK, GEN	14	8	9	7	5	8	-	1	4	2	2	4
CAZ, PIP, AK, GEN	2	1	3	8	3	4	1	1	1	-	-	-
CAZ, PIP, CIP,ATM	-	-	1	1	-	-	-	-	-	-	-	2

CAZ: ceftazidime; PIP: piperacillin; CIP: ciprofloxacin; AK: amikacin; ATM: aztreonam ; GEN: gentamicin; IMP R: imipenem resistant, IMP S: imipenem sensitive,.

[Table/Fig 3]: Mettalo-beta-lactamase detection in different genera.

Organisms	DDST			CDT			E MBL test		
	R	I	S	R	I	S	R	I	S
Acinetobacter baumanii	19	7	6	14	6	5	14	6	5
Pseudomonas aeruginosa	21	5	4	17	5	2	17	5	2
E. coli	1	1	1	1	1	1	1	1	1
Klebsiella pneumoniae	1	2	1	-	1	-	-	1	1

DDST: Double disc synergy test, CDT: Combined disc test, E MBL: E strip test for Metallobeta lactamases, IMP R: imipenem resistant, IMP I: imipenem intermediate, IMP S: imipenem sensitive.

DISCUSSION

The increasing prevalence of MBL-producing gram-negative bacilli in many geographical regions and their propensity to rapidly disseminate within an institution, makes it essential to detect MBL- producing isolates by simple and rapid phenotypic methods.[1],[2] Unfortunately, MBL production is just not limited to carbapenem resistant strains, but has also been demonstrated in some carbapenem susceptible isolates.[11],[12] These organisms carrying hidden MBL genes, may spread unnoticed and may lead to untoward infection control problems.[3] Screening of only carbapenem-resistant organisms is insufficient and screening of all the IMP susceptible isolates for MBL creates unnecessary work with a lower yield. Hence, some criteria is needed to select out IMP susceptible isolates for MBL screening, as has been suggested by some other workers.[11]

In the present study, all the isolates which were screened were multidrug resistant (resistant to 4 or more classes of antimicrobials), as shown in Table 2. 68% of the IMP resistant isolates were susceptible to aztreonam, a profile which was compatible with MBL production. However 32 % of IMP resistant isolates revealed full or intermediate resistance to aztreonam, suggesting the co-existence of another mechanism of resistance among these isolates, most importantly ESBL or the AmpC-type ß-lactamases. Similar findings were also reported by Franklin *et al* who found that 37% of the MBL-carrying isolates were resistant to aztreonam.[11]

The inhibition of enzyme activity by EDTA and thiol compounds was an important characteristic which was used to distinguish MBLs from other ß-lactamases, but MBL detection was difficult among imipenem susceptible isolates by using IMP, as MBLs are inhibited by low concentrations of IPM.[11],[12] CAZ has been recommended by many authors to screen MBL producers, but the MBL-producing strains may also have another CAZ resistance mechanism, thus having a chance to result in false positive results.[6],[11],[16] Hence, we detected MBL in both IMP resistant and susceptible isolates by using IMP EDTA.

In the present study, we utilized the CDT (IMP and IMP EDTA), DDST (IMP and EDTA) and MBL Etest strip methods in an attempt to detect such challenging organisms. 2-mercaptopropionic acid and 1, 10-phenanthroline were not used to inhibit MBLs, as these are toxic for routine handling and required special precautions.[11]

In our study, 20% of the MBL carrying isolates were found to be susceptible to IMP as against a very high rate varying from 30-88%, which was reported by other workers.[11],[12] There was 100% concordance between the CDT and MBL E test strips. DDST detected more number of isolates as MBL producers, as even a slight increase in the synergistic zone was taken as positive. This could have been due to a zone difference of ≥7 mm which was taken as positive by the IPM-EDTA method as compared with using IPM alone in the CDT

method. Had a cut off of ≥ 4mm been chosen for MBL detection, as done by some workers, some additional isolates would have been screened to be positive for MBLs by CDT.[11] However, all the isolates with an increase in the zone diameter of less than 7mm were screened to be negative for MBLs by the MBL Etest strip method, suggesting that increase in the zone diameter of ⩾7 mm is an acceptable cut off with 750 µg EDTA.

In this study, 6 MBLs carrying IMP sensitive isolates had a zone diameter in between 16-20 mm and 2 isolates had a zone diameter between 21-22 mm. No IMP sensitive isolate with a zone diameter ≥ 23 mm was detected as an MBL producer, thus indicating that all the isolates with a zone diameter ≤22 mm should be routinely screened for MBL production.

Thirty eight (63%) IMP R isolates were detected as MBL producers by all the three methods. Among other IMP resistant isolates, carbapenem resistance may be due to other mechanisms of resistance such as decreased permeability of the outer membrane and/or active efflux, which is possibly associated to the overproduction of the endogenous class C ß-lactamases.[16],[17]

Several phenotypic methods have been evaluated by various workers by using different combinations of antibiotic disks along with different concentrations of EDTA and different inoculum sizes of the test strain.[5-7] No single method has been proven as an ideal method for MBL detection in all the isolates and so, we used the three most user friendly techniques for MBL detection. Our results demonstrated that the combined-disk test was a better method for screening purposes as it was simple to perform and the materials used were cheap, nontoxic, easily accessible and allowed for the objective interpretation of results. Moreover, this method was quite good in detecting carbapenem-susceptible MBL-producing isolates, a phenotype that is being described with increasing frequency. Therefore, it is recommended that MBL detection must be done in all diagnostic laboratories on a daily basis to prevent the emergence and spread of this worrying resistance mechanism.

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