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Phenotypic detection of metallo-β-lactamase among the clinical isolates of imipenem resistant *Pseudomonas* and *Acinetobacter* in tertiary care hospitals

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Abstract

Background: The rapid spread of Metallo-β-lactamases (MBLs) producing Gram negative bacilli especially *Pseudomonas aeruginosa* and *Acinetobacter* species represents a matter of great concern worldwide. The present study was undertaken to analyze the occurrence of MBLs production in Imipenem resistant *Pseudomonas* and *Acinetobacter ssp*. isolates.

Method: Non fermentative Gram Negative bacilli (NFGNB) were isolated from a variety of clinical specimens, plated on blood agar and MacConkey agar and the *Acinetobacter* and *Pseudomonas species* were identified by standard microbiological techniques. The Imipenam resistant strains were subjected to MBL detection by double disk synergy test, (DDST), combined disk test (DPT) and minimum inhibitory concentration (MIC) reduction test.

Result: A total of 250 *Acinetobacter spp* and 180 *Pseudomonas spp* isolates were obtained from Tertiary Care Hospitals during the period of two years. 44 (17.6%) *Acinetobacter* strains and 24 (13.3%) *Pseudomonas* strains were resistant to Imipenem. Amongst 44 screen test positive isolates of *Acinetobacter spp*., MBL production was detected in 34 (77.27%) isolates each by DDST and MIC reduction test, in 32 (72.72%) isolates by DPT while amongst 24 positive *Pseudomonas spp*. stains, MBL production was detected in 20 (83.33%) isolates each by DDST and MIC reduction test, in 19 (79.16%) isolates by DPT.

Conclusion: MIC reduction and DDSM were better methods for the detection of MBL as compared to DPT. All the microbiology laboratories must routinely screen for imipenem resistance due to MBL, which will help to reduce morbidity and mortality in the patients. Selected use of imipenem and infection control programs for nosocomial infection should also be practiced.

Keywords: Metallo-β-lactamases, *Pseudomonas aeruginosa*, *Acinetobacter*, Imipenem, Resistant

Introduction

Non-fermenting Gram-negative bacilli (NFGNB) are a group of aerobic, non-sporing organisms that either does not use carbohydrates as a source of energy or consume them through metabolic pathways other than fermentation [1]. These organisms are abundant in nature and cause severe infections in immune-compromised hosts [2]. Most of the NFGNB such as *Acinetobacter* species and

Pseudomonas aeruginosa have emerged as important nosocomial pathogens causing nosocomial infections [3-5]. These organisms are generally intrinsically resistant to a wide variety of antimicrobial agents as well as they have the capacity to develop resistance by mutation or acquisition of foreign resistance genes against different antibiotic classes [6].

Carbapenem, including imipenem, meropenem and doripenem are often used as a last resort for treatment of infections caused by *P. aeruginosa*, *Acinetobacter* species and other Gramnegative bacteria [7, 8]. Carbapenem is a β lactam antibiotic with a broad spectrum antibacterial activity and is stable to almost all clinically relevant extended spectrum beta lactamases. But unfortunately resistance to these antibiotics started emerging and has been reported worldwide among NFGNB specially *Pseudomonas* and *Acinetobacter* isolates [9]. This resistance to carbapenem is due to decreased outer membrane permeability, increased efflux systems, alteration of penicillin binding proteins and carbapenem hydrolyzing enzymes carbapenemase [10]. Among carbapenemases transferable Metallo-β-lactamases (MBL) are the most feared because of their ability to hydrolyze all β – lactams, including carbapenems, except monobactams.

MBLs spread easily via plasmids and cause nosocomial infections and outbreaks. Therefore, the rapid detection of MBL – producing GNB is necessary to aid infection control and to prevent dissemination. MBLs are metalloenzymes of Ambler class B and are clavulanic acid-resistant enzymes. They require divalent cations of zinc as co-factors for enzymatic activity and are universally inhibited by ethylenediamine tetra-acetic acid (EDTA), as well as other chelating agents of divalent cations [11]. Therefore several laboratory methods using metal chelators as EDTA have been described for the phenotypic detection of MBLs among clinical isolates [4, 12].

In view of the above, the present study was undertaken to determine the prevalence of MBLs production in Imipenem resistant *Pseudomonas* and *Acinetobacter ssp*. Isolates during the study period of two years.

Materials and Methods

The present study was done in the Department of Microbiology, during the period of two years. All the 440 isolates from urine, pus, wound swabs, sputum, ET aspirate, blood and ascitic fluid were obtained from the patient admitted in ICU, ward and outpatient department of Tertiary Care Hospital. All clinical samples were cultured on blood agar and MacConkey agar and incubated at 37°C for 18–24 h under aerobic conditions. NFGNB were identified by standard microbiological techniques [13,14] by studying their morphology [15], colony characteristics [13] and biochemical reactions [16]. These isolates were subjected to antimicrobial susceptibility testing as per CLSI 2014 guidelines [17] by Kirby-Bauer disk diffusion technique [18]. The Imepenam resistant strains were subjected to MBL detection by Double disk synergy test, combined disk test/ Disk Potentiation Test and Minimum inhibitory concentration reduction test.

Double disk synergy test [19]: A 0.5 McFarland inoculum of the test strain was spread over MHA. Imipenem (10 µg) and meropenem (10 µg) disks were placed 4-5 cm apart from centre to centre from ceftazidime (30 µg) disk. A blank filter paper disk was place near the ceftazidime disk, 15-20 mm apart from each other. 5 µl EDTA was added to the blank filter paper disk onto the agar plate and incubated at 37oC for 16-20 hours. Since EDTA may have some bactericidal activity, a

blank disk of EDTA was tested as control [20]. Presence of even a small synergistic inhibition zone was interpreted as positive.

Combined disk test [21]: In this test, the lawn culture of 0.5 McFarland inoculum of the test strain was exposed to a disk of imipenem (10 µg) and imipenem-EDTA (10/750 µg). The difference of ≥7mm in zones of inhibitions of two disks indicated MBL production.

Minimum inhibitory concentration reduction testing [22]: A minimum fourfold reduction in MIC of the strains when tested in combination with EDTA as compared to MIC for imipenem alone, confirmed that the strains were MBL producers. Material required for this test were 1) Medium: Muller Hinton agar, 2) Antimicrobial powders (Imipenem, EDTA) (HiMedia Lab, Mumbai) of known potency and solvents and diluents.

Antibiotic	Solvent	Diluent
Imipenem	Phosphate buffer, pH 7.2, 0.01 mol/l	Phosphate buffer, pH 7.2, 0.01 mol/l
EDTA 1 ml solution (0.5 M) was added to 1 ml of imipenem spanning similar concentrations as done for MIC to imipenem		

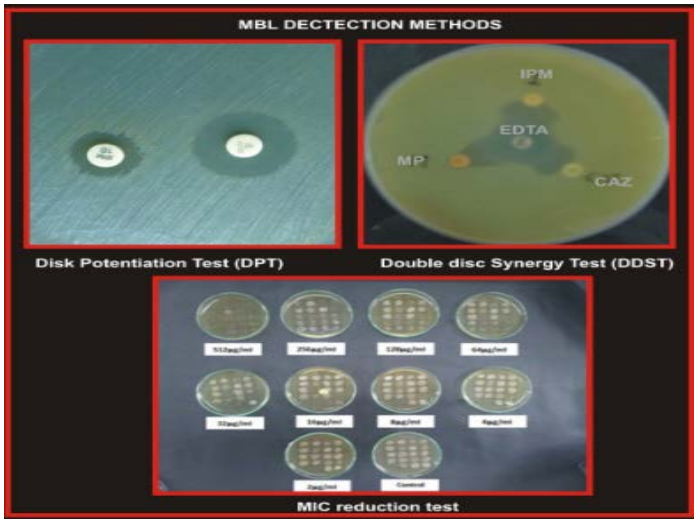
Solutions were used immediately for preparation of agar.

Dilutions Used

Imipenem	1, 2, 4, 8, 16, 32, 64, 128, 256, 512µg/ml
Imipenem-EDTA	1, 2, 4, 8, 16, 32, 64, 128, 256, 512µg/ml

Dilutions were made by using corresponding diluents. This was then added to 25 ml of molten and cooled agar with temperature not more than 60^{o}C. Each 2 ml of EDTA and Imipenem solution was added to 25 ml of molten MHA and poured on plates that were allowed to set. These plates were used within 24 hours of preparation. The inoculum was matched with 0.5 McFarland standards. The reverse of the plate was divided into small squares. Each square was spot inoculated with a fixed inoculum of 10^{7}cfu/ml. Then the plates were incubated at 37^{o}C for 18–24 hours. For the quality control- 1) *E. coli* ATCC 29212 and *P. aeruginosa* ATCC 27853 were inoculated on all plates of different antibiotic concentration, 2) A plate of MHA without antibiotic was inoculated with all strains to be tested. All strains grown on control plate indicated that all strains were alive.

Hazy growth and 1 or 2 colonies on the spot were ignored. Only the prominent growth was taken into account. MIC was interpreted as follows; e.g. the concentrations of imipenem used were: 1, 2, 4, 8, 16, 32, 64, 128, 256, 512µg/ml. The highest dilution that inhibited the growth of the organism was taken as MIC. If the strain grew on only first nine plates and there was no growth on tenth plate then the MIC of the strain would be 256µg/ml. The results were interpretation as susceptible, intermediate and resistant as per CLSI 2014 guidelines by using MIC interpretive criteria [13]. A minimum fourfold reduction in MIC of these strains when tested in combination with EDTA as compared to MIC for imipenem alone, confirmed that the strains were MBL producers [22].



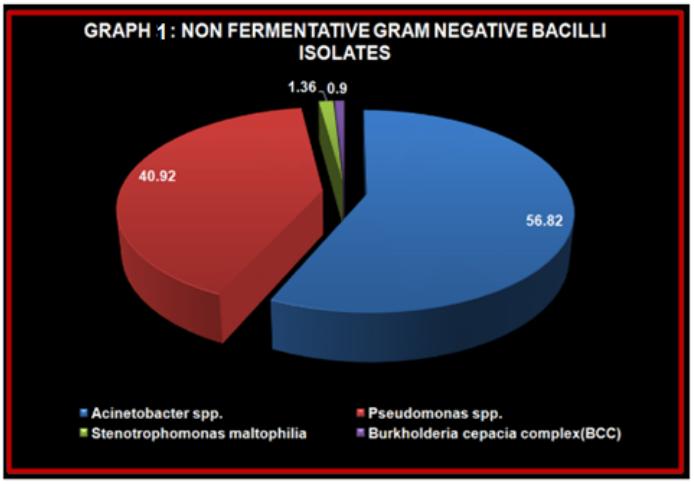
Statistical Analysis

Chi square test was used with appropriate correction to see the significance of difference between the sensitivity of Imipenem in MBL producing strains using SPSS software. $p \leq 0.05$ was considered significant.

Observations and Results

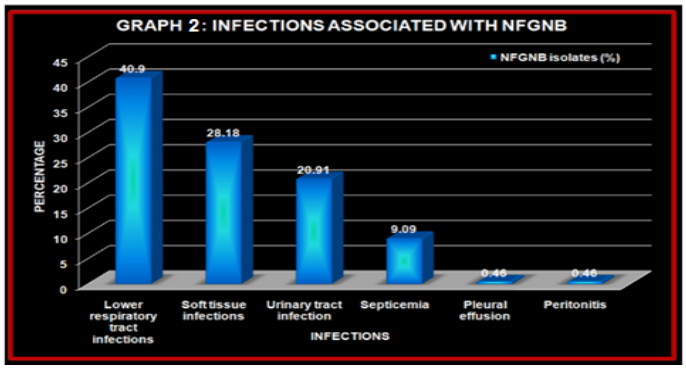
A total of 8,468 clinical samples were collected during the two years of study period from various wards. Out of the total 8,468 samples processed, 234 (20.24%) isolates were from intensive care units, 129 (2.4%) from inpatient department and 77 (3.94%) from outpatient department, (p value < 0.05). Thus, total 440 NFGNB were isolated from 8,468 clinical specimens. Out of 440 isolates, most frequently isolated NFGNB were *Acinetobacter spp.* (56.82%) followed by *Pseudomonas spp* (40.92%), as shown in graph 1 and which were selected for the study.

Graph 1: Non fermentative gram negative bacilli isolates



The most common infections caused by NFGNB were lower respiratory tract infections (40.90%) followed by soft tissue infections (28.18%), urinary tract infections (20.91%) and septicemia (9.09%). NFGNB were responsible for significantly higher respiratory tract infections, (p-value<0.05), (Graph 2).

Graph 2: Infections associated with NFGNB



Among the total 250 *Acinetobacter spp* isolates, 44 (17.6%) strains were resistant to Imipenem whereas among 180 *Pseudomonas spp* isolates, 24 (13.3%) *Pseudomonas* strains were resistant to Imipenem. There was no statistically significant difference (p value > 0.05) in the MBL production by DPT and DDST in both the Imipenem resistant *Acinetobacter spp.* and *Pseudomonas* isolates as shown in table 1. Also, it is seen in the table that DDST method is better than DPT in detecting MBL producer which missed two MBL producers in *Acinetobacter spp* and one MBL producers in *Pseudomonas spp*. None of the isolates of *Stenotrophomonas maltophilia* and *Burkholderia cepacia complex* were resistant to imipenem. Hence none were further subjected for MBL production.

Table 1: MBL production by DPT and DDST among Imipenem resistant *Acinetobacter spp.* (n=44) and *Pseudomonas* isolates (n=24)

Method	MBL production				P value
	Among Imipenem resistant *Acinetobacter spp* (n=44)		Among Imipenem resistant *Pseudomonas spp* (n=24)		
	DPT (%)	DDST (%)	DPT (%)	DDST (%)	
No. of strains showing MBL production	32 (72.72)	34 (77.27)	19 (79.16)	20 (83.33)	> 0.05

With Imipenem (IMP), the highest MIC shown by *Acinetobacter* isolates was 256 µg/ml (11.36%) followed by 128 µg/ml (22.73%), 64 µg/ml (34.09. %), 32 (18.18%) µg/ml, 16 (4.55) and the lowest was 8 µg/ml (9.09%). With IMP, the highest MIC shown by *Pseudomonas* isolates was 512 µg/ml (4.16%) followed by 256 µg/ml (12.5%), 128 µg/ml (29.16%), 64 µg/ml (25.00. %), 32 µg/ml (16.67%) and the lowest was 16 µg/ml (12.5%), (Table 2).

Table 2: MIC of Imipenem screen test positive *Acinetobacter* isolates and *Pseudomonas* isolates by Agar Dilution method

Imipenem MIC (µg/ml)	No. of *Acinetobacter* isolates (%)	No. of *Pseudomonas* isolates (%)
512	-	1(4.16)
256	5 (11.36)	3(12.5)
128	10 (22.73)	7(29.16)
64	15 (34.09)	6(25.00)
32	8 (18.18)	4(16.67)
16	2(4.55)	3(12.5)
8	4(9.09)	-
4	-	-
2	-	-
1	-	-
Total	44	24

MIC for Imipenem: Resistant = ≥ 8 µg/ml, Sensitive = ≤ 2 µg/ml

MBL *Acinetobacter* confirmation- 34 screen test positive isolates showed fourfold or more (4-32 folds) fall in MICs with IMP in the presence of mixture of EDTA as compared to IMP alone. 10 isolates showed less than fourfold reduction in MIC. The MIC in presence of mixture of EDTA ranged between 1-32 µg/ml as compared to MIC with IMP alone (8-256 µg/ml), (Table 3).

MBL *Pseudomonas* confirmation- 20 screen test positive isolates showed fourfold or more (4-32 folds) fall in MICs with IMP in the presence of mixture of EDTA as compared to IMP alone. 4 isolates showed less than fourfold reduction in MIC. The MIC in presence of mixture of EDTA ranged between 1-32 µg/ml as compared to MIC with IMP alone (16-512 µg/ml), (Table 3).

Table 3: Reduction in MIC of Imipenem in presence of mixture of EDTA for MBL *Acinetobacter* confirmation and MBL *Pseudomonas* confirmation

MBL *Acinetobacter* confirmation				MBL *Pseudomonas* confirmation			
No. of isolates	IMP MIC	MIC of IMP in presence of EDTA	MIC reduction (in folds)	No. of isolates	IMP MIC	MIC of IMP in presence of EDTA	MIC reduction (in folds)
1	256	32	8	1	512	16	32
2	256	16	16	2	256	32	8
2	256	8	32	1	256	8	32
2	128	32	4	2	128	16	8
3	128	16	8	2	128	8	16
4	128	8	16	4	128	4	32
1	128	4	32	3	64	32	2
4	64	32	2	2	64	16	4
3	64	16	4	1	64	8	8
5	64	8	8	1	32	16	2
2	64	4	16	2	32	2	16
1	64	2	32	1	32	1	32
4	32	16	2	2	16	2	8
3	32	2	16	1	16	1	16
1	32	1	32				
1	16	8	2				
1	16	1	16				
3	8	2	4				
1	8	4	2				

All the 34 (77.27%) *Acinetobacter* isolates positive for MBL production by MIC reduction and DDST. DPT showed MBL production in 32 (72.72%) isolates, (Table 4). However, the difference was not statistically significant, (p value > 0.05). Also, table 4 shows that all the 20 (83.33%) *Pseudomonas* isolates positive for MBL production by MIC reduction and DDST. DPT showed MBL production in 19 (79.16%) isolates. However, the difference was not statistically significant (p value > 0.05).

Table 4: Comparison of methods of MBL detection in *Acinetobacter* spp and in *Pseudomonas aeruginosa*

Method	MBL production					
	Among Imipenem resistant *Acinetobacter spp* (n=44)			Among Imipenem resistant *Pseudomonas spp* (n=24)		
	DPT (%)	DDST (%)	MIC reduction	DPT (%)	DDST (%)	MIC reduction
No. of strains showing MBL production	32 (72.72)	34 (77.27)	34 (77.27)	19 (79.16)	20 (83.33)	20 (83.33)

Discussion

As MBLs will hydrolyze virtually all classes of β-lactams, there continued spread will be a clinical catastrophe [11]. With the global increase in the types of MBLs, an early detection is crucial [11, 23]. CLSI has not laid Performance standards with no standard guidelines for detection of MBL. So a number of screening methods had been employed in different studies [4, 24]. In the present study, MBL production was detected by using Double disk synergy test (DDST), combined disk test/ disk potentiating test (DPT) and minimum inhibitory concentration (MIC) reduction test. Though MIC detection is the gold standard phenotypic test, DDST and DPT are comparable with the former and at the same time, are simple, reliable, less cumbersome and cheap, as per previous reports [4, 25, 26].

The present study reported 17.6% Imipenem resistance among *Acinetobacter* strains which is similar to study done by Sinha et al [27]. However higher resistance rates have been reported by previous studies [28, 29]. Imipenem resistance among *Pseudomonas* was observed in 13.3% which is comparable with the study done by Shivappa et al [30] they have reported 15.3% Imipenem resistance among *Pseudomonas* strains. The high rate of Carbapenem resistance have also been reported by Varaiya et al (25%) [10] and Noyal et al (31.1%) [28]. Indiscriminate and irrational use of antibiotics has led to increase in the incidence of Carbapenem resistance among *Pseudomonas* and *Acinetobacter* species. MBL production was detected in 32 (72.72%) by DPT and 34 (77.27%) by DDST in Imipenem resistant *Acinetobacter* isolates. While amongst Imipenem resistant *Pseudomonas* isolate DPT detected 19 (79.16%) MBL producers whereas DDST detected 20 (83.33%) MBL producers isolates. These findings are correlated with the other studies [31, 32].

The minimum inhibitory concentration (MIC) values for imipenem amongst the *Acinetobacter* isolates were ≥ 8 µg/ml. 34 screen test positive isolates showed fourfold or more (4-32 folds) fall in MICs with IMP in the presence of mixture of EDTA as compared to IMP alone. 10 isolates showed less than fourfold reduction in MIC. Of the 44 imipenem resistant *Acinetobacter* strains, 34 strains were MBL producers. The remaining isolates may possess other enzymes mediating carbapenem resistance, such as OXA-typeβ-lactamases (class D) and/or other mechanisms such as outer-membrane permeability and efflux mechanisms that were not checked [33]. A study by Rit et al [34] showed 22% *Acinetobacter* strains to be MBL producers by reduction of MIC value by four fold or more after addition of EDTA to imipenem.

Moreover, the MIC values for imipenem amongst the 24 isolates of *Pseudomonas* were ≥ 16 µg/ml. 20 screen test positive isolates were showed fourfold or more (4-32 folds) fall in MICs with IMP in the presence of mixture of EDTA as compared to IMP alone. Four isolates showed less than fourfold reduction in MIC. In a study by Buchunde et al [35] all the 58 (100%) IPM resistant screen-test positive isolates of *Pseudomonas aeruginosa* showed four-fold or more fall in MICs of IPM in the presence of mixture of EDTA as compared to IPM alone. A study by Rit et al [34] showed 41% *Pseudomonas* strains to be MBL producers by reduction of MIC value by four fold or more after addition of EDTA to imipenem.

When comparing all the three test used for the detection of MBL production, we found that amongst 44 screen test

positive isolates of *Acinetobacter spp*., MBL production was detected in 34 (77.27%) isolates each by double disk synergy test and MIC reduction test, in 32 (72.72%) isolates by combined disk test. While amongst 24 screen test positive isolates of *Pseudomonas spp*., MBL production was detected in 20 (83.33%) isolates each by double disk synergy test and MIC reduction test, in 19 (79.16%) isolates by combined disk test. These findings are correlated well with the earlier studies [34-36].

Conclusion

With the increasing use of carbapenems (imipenem) for treating infections, the problem of MBL production is also increasing. Even though MBL production is an important mechanism of Carbapenem resistance among *Pseudomonas* and *Acinetobacter* species and other mechanisms are also seen at a higher rate. In the present study, MIC reduction and double disk synergy methods for detection of MBL were better as compared to disk potentiation test. MBL producing isolates were multidrug resistant making therapeutic choices limited. Polymyxin B and Colistin are the next therapeutic options for carbapenem resistant isolates. Continuous antibiotic surveillance, infection control practices and an effective antibiotic policy are required to address the problem of MBL – associated infections. Molecular studies are necessary to evaluate the various MBL type. The present study suggested to provide correct antibiotics to the patients infected with MBL producer and to prevent their spread. All the microbiology laboratories must routinely screen for imipenem resistance due to MBL, which will help to reduce morbidity and mortality in these patients. Selected use of imipenem and infection control programs for nosocomial infection should also be practiced.

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