International Journal of Research in Medical Sciences
Mehta A et al. Int J Res Med Sci. 2016 Sep;4(9):4084-4088
www.msjonline.org
pISSN 2320-6071 | eISSN 2320-6012

DOI: http://dx.doi.org/10.18203/2320-6012.ijrmss20162938

Research Article

Detection and characterization of metallo-β-lactamases producing *Pseudomonas aeruginosa* clinical isolates at a tertiary care hospital of Bhopal, India

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Received: 13 July 2016
Accepted: 08 August 2016

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ABSTRACT

Background: Carbapenems are very often used effectively against multidrug resistant strains of clinically troublesome pathogens like *P. aeruginosa*. But there is emerging threat of developing resistance against carbapenems mainly attributed to metallo-β-lactamases production which has proved to be a clinical disaster. Early detection and identification of MBL-producing strains is of crucial importance for the prevention of nosocomial dissemination through appropriate treatment, as well as the timely implementation of infection control measures. Therefore this study was undertaken for screening MBL production in clinical isolates of *Pseudomonas aeruginosa.*
Methods: One hundred fifty consecutive clinical isolates of *P. aeruginosa* were subjected to susceptibility testing by disc-diffusion test as per CLSI 2014 guidelines. Imipenem resistant strains were then subjected to screening for MBL production by Imipenem- EDTA combined disc test and Imipenem-EDTA double-disc synergy test.
Results: A total of 20 (13.3%) isolates of *P. aeruginosa* showed resistance to Imipenem out of which twelve (8%) were screened as MBL producers by CDT whereas 8 (5.3%) isolates gave positive result by DDST.
Conclusions: Introduction of simple, cheap, reliable, reproducible screening tests for early detection and identification of MBL-producing organisms in routine diagnostics is of crucial importance for the prevention of nosocomial dissemination of MBL through appropriate treatment, as well as the timely implementation of effective infection control measures. Antibiotic Stewardship and strict antibiotic policy enforcing judicious use of antibiotics is the need of hour.

Keywords: *Pseudomonas aeruginosa*, Carbapenems, Metallo beta lacatamases, Imipenem-EDTA (Ethylene di amine tetra acetate) Combined disc test, Double disc synergy test

INTRODUCTION

Pseudomonas aeruginosa is well known as a clinically troublesome pathogen causing a wide range of opportunistic infections and nosocomial outbreaks.[1] Carbapenems are often used as antibiotics of last resort for treating infections due to multi-drug resistant Gram-negative bacilli.[2] Resistance to carbapenems is due to impermeability via the loss of the OprD porin, the up-regulation of an active efflux pump system of the cytoplasmic membrane, alteration of penicillin binding proteins or the production of metallo-β- lactamases (MBLs).[3] These enzymes belong to Ambler class B and Bush group 3 and require divalent cations, usually Zinc,

as a cofactor for enzyme activity and are inhibited by metal chelators such as EDTA.[3,4]

Metallo-β-lactamase (MBL) producing *P. aeruginosa* is an emerging threat and a cause of concern for treating physicians.[5] Acquired MBL in *Pseudomonas spp.* have recently emerged as one of the most worrisome resistance mechanism because of their capacity to hydrolyze all beta (β)-lactam antibiotics including penicillins, cephalosporins and carbapenems, with the exception of aztreonam.[2]

Moreover, MBLs are not susceptible to therapeutic β-lactamase inhibitors like sulbactam, tazobactam, or clavulanic acid and no new inhibitor of these enzymes is yet in the pipeline, hence their continued spread would be a clinical disaster. This situation prompts an early and accurate detection of MBL producing organisms of crucial importance like non-fermenting pathogens.[6]

The worldwide dissemination of acquired metallo-beta-lactamases genes and the emergence of new variants are becoming an emerging threat to public health because they usually are carried by mobile genetic elements that disseminate rapidly. Therefore, early detection and identification of MBL-producing organisms is of crucial importance for the prevention of nosocomial dissemination through appropriate treatment, as well as the implementation of infection control measures.[7]

There is not much information available on MBL producing *P. aeruginosa* isolates from this part of India. In the light of above facts we therefore undertook this study to detect MBL production in clinical isolates of *Pseudomonas aeruginosa.*

METHODS

This Prospective Analytical study was carried out at the Dept. of Microbiology of a Tertiary care Teaching Hospital of Central India from April 2015 to November 2015 after obtaining Ethical clearance from Institutional Ethics Committee.

Study period and clinical samples

Samples like blood, urine, sputum, wound swabs, catheter tips, ET tips, pus, HVS and other body fluids obtained from patients admitted in Peoples hospital, Bhopal submitted to Dept. of Microbiology for routine diagnostic workup between April 2015 to November 2015 were processed as per the standard Protocol. Identification and characterisation was done by standard microbiological techniques.[8]

Inclusion criteria

A total of 160 consecutive isolates of *P. aeruginosa* obtained from various clinical samples of admitted patients over a period of eight months (April- Nov.2015) were included in the study.

Non repetitive clinical isolates of *P. aeruginosa* from in-patients, which were found to be resistant to Imipenem, were selected for further characterization.

Antimicrobial susceptibility test[9]

Antimicrobial susceptibility Test of all 160 clinical isolates of *P. aeruginosa* was performed on Mueller Hinton Agar by Kirby Bauer disc diffusion method in accordance with CLSI-2014 guidelines incorporating standard strain of *P. aeruginosa* (ATCC 27853). The antibiotics tested were Amikacin, Gentamycin, Cefepime, Ceftazidime, Ceftriaxone, Cefoperazone, Ciprofloxacin, Amoxycillin clavulanate, Piperacillin tazobactum, Imipenem, Polymixin B and Aztreonam. EDTA, extra pure (Hi-media Laboratories, Mumbai) powder was used for screening MBL production *P. aeruginosa* strains were considered Carbapenem resistant, when the zone size around Imipenem disc ≤ 13 mm, intermediate 14-15 mm and sensitive ≥ 16 mm (CLSI-2014). Imipenem resistant isolates were further screened for MBL production.

Phenotypic Tests for MBL Detection[2-6,10-14]

1) Imipenem- EDTA combined disc test (CDT)

The Imipenem- EDTA Combined disc test (CDT) was performed as described by Lee et al. A 0.5 M EDTA solution was prepared by dissolving 18.61g of Disodium EDTA**.** $2H_2O$ in 100 ml of distilled water and adjusting pH to 8.0 using NaOH. The mixture was sterilised by autoclaving. Direct colony suspension of test organism adjusted to match 0.5 McFarland turbidity was prepared and inoculated onto Mueller-Hinton agar plate. Two imipenem (10ug) discs placed on the surface of agar plate at a distance of 25 mm and 4 ul EDTA solution added to one of the disc to obtain a desired concentration of 750 ug. The inhibition zones of imipenem and imipenem-EDTA discs compared after 16 to 18 h of incubation in air at 37^oC. In the combined disc test, if the increase in inhibition zone with imipenem- EDTA disc came out to be ≥7 mm than the imipenem disc alone, then it was considered as MBL positive.

2) Imipenem - EDTA double- disc synergy test (DDST) Imipenem-EDTA Double Disc Synergy Test (DDST)

The Imipenem-EDTA Double Disk Synergy Test was performed as described by Lee et al. An imipenem (10 µg) disc was placed 20 mm centre to centre from a blank disc containing 4 µl of 0.5 M EDTA (750µg). The inhibition zones of the Imipenem and EDTA discs were compared after 16 to 18 hrs of incubation at 37^0C. Enhancement of the zone of inhibition in the area between Imipenem and the EDTA disc in comparison with the zone of inhibition on the far side of the

Imipenem disc was interpreted as a positive result when this enhancement ≥5mm.

RESULTS

A total of 150 consecutive Non-repetitive isolates of *Pseudomonas aeruginosa* obtained from various clinical samples over a period of eight months (April-Nov.2015) were included in the study out of which 59 were isolated from pus, 47 from sputum, 15 from urine, 11 from blood, 9 from ET tip, 6 from Foleys Cathetre tip, 2 from bronchial washings and 1 from High Vaginal Swab (HVS) as depicted in Table 1.

Table 1: Sample wise distribution of *P. aeruginosa* isolates.

Samples	*P. aeruginosa* isolates (150)	Carbapenem Resistant isolates (20)	MBL Producers (12)	
			By CDT (12)	By DDST (8)
Pus	59	10	7	5
Sputum	47	4	1	1
Urine	15	2	2	1
Blood	11	1	0	0
ET tip	9	2	1	0
Foleys Cath. tip	6	1	1	1
Bronchial washings	2	0	0	0
HVS	1	0	0	0

MBL-Metallo β-lactamase CDT-Combined Disc Test DDST-Double disc synergy Test

Out of 150 isolates of *P. aeruginosa* 110 (73.33%) were found to be resistant to Ceftriaxone, 103 (68.67%) to Cefoperazone, 100 (66.67%) to Cefepime, 90 (60%) to Amoxycillin clavulanate, 75 (50%) to Ciprofloxacin, 60 (40%) to Amikacin, 40 (26.67%) to Piperacillin tazobactum, 30 (20%) to Ceftazidime, 20 (13.3%) to Imipenem, 15 (10%) to Aztreonam and 2 (1.3%) to Polymixin B.

A total of 20 (13.3 %) isolates of *P. aeruginosa* showed resistance to Imipenem which were then tested for metallo betalactamase (MBL) production by Imipenem-EDTA Combined Disc Test (CDT) and Double disc Synergy Test (DDST).Twelve of these 20 isolates exhibited a ≥7mm. zone size enhancement in CDT whereas 8 isolates gave positive result by DDST.

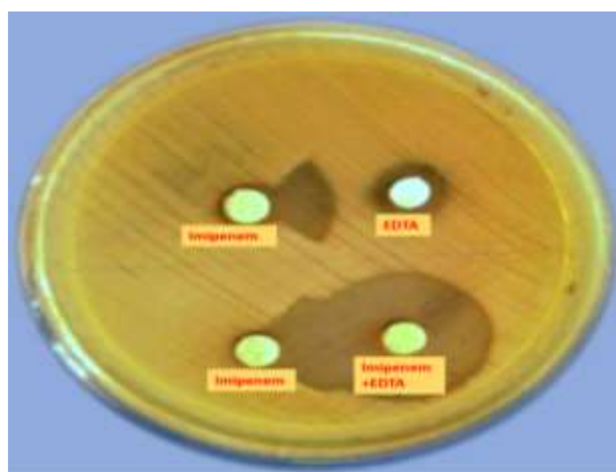
Those isolates which were found MBL positive by DDST were also found to be MBL positive by CDT.

The ATCC 27853 *P. aeruginosa* did not exhibited any zone size enhancement with EDTA impregnated Imipenem discs (CDST).

Table 2: Antibiotic sensitivity pattern of carbapenem resistant *P. aeruginosa* strains with reference to MBL production.

Antibiotics	Carbap-enem resistant strains (20)	MBL +ve (12)	MBL -ve (8)
Amikacin (30)	6	2	4
Gentamicin (10)	2	0	2
Cefepime (30)	2	0	2
Ceftazidime (30)	3	0	3
Ceftriaxone (30)	0	0	0
Cefoperazone (75)	0	0	0
Amoxycillin clavulanate	4	0	4
Piperacillin tazobactum	8	2	6
Ciprofloxacin (5)	2	0	2
Imipenem (10)	0	0	0
Aztreonam	15	7	8
Polymixin B	18	10	8

MBL- Metallo-β-lactamase



CDT-Combined disc test; DDST-Double disc synergy test.

Figure 1: Screening of metallobetalactamase production by imipenem-EDTA DDST (upper half) and imipenem-EDTA CDT (Lower half).

In this study we found Ceftazidime to exhibit 80% sensitivity against all clinical isolates of *P. aeruginosa* but only 15% sensitivity against Carbapenem resistant strains with no sensitivity against MBL producer strains. In this study we had found Polymixin B as the most effective antibiotic with 98.67% (148/150) sensitivity against clinical isolates of *P. aeruginosa*, 90% (18/20) sensitivity against Carbapenem resistant strains and 83.33% (10/12) sensitivity against MBL producer strains followed by Aztreonam with 90% (135/150) sensitivity against *P. aeroginosa* isolates, 75% (15/20) against Carbapenem resistant strains and 58.33% (7/12) against MBL producers.

DISCUSSION

Carbapenems had proved to be the most potent agents against multidrug resistant gram negative bacterial infections. But there is an alarming increase in reports of growing resistance to such life-saving antimicrobials in *Pseudomonas aeruginosa.*[6,12,15,16]

Carbapenem hydrolysing Metallo beta lactamases production had emerged as the most important mechanism behind Carbapenem resistance. This has tremendous therapeutic significance as these bacteria also carry other multi drug resistance genes and are found to be resistant to many antibiotic groups like beta-lactams, aminoglycosides, fluoroquinolones and only therapeutic options left are Polymixin B and Colistin which carry potential toxicity.[6,15]

The continuing spread and increasing prevalence of MBL has proved to be a clinical disaster and pose a serious challenge to infection control management due to their role in unnoticed spread within institutions and their intrinsic capability to participate in horizontal MBL gene transfer with other pathogens in the hospital settings. Early identification of such infections is necessary as appropriate and timely treatment might reduce the mortality and hospital stay.[17]

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.[6,15] In this study we had used 2 conventional Phenotypic tests Imipenem-EDTA Combined Disc Test (CDT) and Double Disc Synergy Test (DDST). Although MIC detection is a Gold standard for MBL detection but CDT and DDST are comparable to it and are also simple, reliable, inexpensive and reproducible.[15,18]

We had found that with Imipenem-EDTA CDT, the positives and negatives could be clearly demarcated but with DDST it was not so due to subjective variations. DDST depends on expertise in discriminating true synergism. So, CDT using Imipenem-EDTA had an edge over DDST. This finding is in accordance with other studies which had found CDT to be one of the most sensitive technique for detecting MBL.[15,19,20]

Some researchers recommend Ceftazidime disc instead of Imipenem disc in CDT/DDST. But as there may be other Ceftazidime resistance mechanisms in MBL producers CDT/DDST using Ceftazidime discs will not detect MBL production and hence Imipenem disc is a better option for screening MBL.[6,15,21]

In India prevalence ranging from 8 to 14% has been reported in number of studies. (Agrawal et al, Mendiratta et al, Navneeth et al, Hemlatha et al, Behera et all, Bashir et al, Varaiya et al, Khakhkhar et al.[4,5,13,15,17,22-24]

In our study out of 150 strains, 12 (8%) were found to be MBL producers. CDT detected higher number of MBL producers (12) than DDST (8). This is in accordance with the study conducted by Behera et al.

CONCLUSION

The worldwide dissemination of acquired metallo-beta-lactamases genes and the emergence of new variants are becoming an emerging threat to public health.

The findings of our study had shown a growing need to conduct regular antibiotic resistance surveillance and to improve and strengthen the infection control practices to curb the dissemination of multi drug resistant strains in hospital settings. Antibiotic Stewardship and strict Antibiotic policy enforcing judicious use of antibiotics for effective control and treatment of such infections is the need of hour.

Introduction of simple, cheap, reliable and reproducible screening tests for early detection and identification of MBL-producing organisms in routine diagnostics is of crucial importance for the prevention of nosocomial dissemination of MBL through appropriate treatment, as well as the timely implementation of effective infection control measures. We recommend introducing a practice of putting an additional EDTA disc (750µgm/ml) on routine AST plates for screening MBL producers.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

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Cite this article as: Mehta A, Tukaram P. Detection and characterization of metallo-β-lactamases producing *Pseudomonas aeruginosa* clinical isolates at a tertiary care hospital of Bhopal, India. Int J Res Med Sci 2016;4:4084-8.