Phenotypic Detection of Metallo-*B*-Lactamases in Imipenem-Resistant Pseudomonas aeruginosa in Hospitalized Patients

M.Shanmugam[a], K.Vishnuvardhanarao[b]

[a]Assistant professor, Department of Microbiology, Dr. Pinnamaneni Siddhartha Institute of Medical Sciences & Research Foundation, Chinoutapally, Gannavaram mandal, Andhra Pradesh, India

[b]Associate professor, Department of Microbiology, Dr. Pinnamaneni Siddhartha Institute of Medical Sciences & Research Foundation, Chinoutapally, Gannavaram mandal, Andhra Pradesh, India

Corresponding author :

M.Shanmugam

Assistant professor, Dr. Pinnamaneni Siddhartha Institute of Medical Sciences & Research Foundation, Chinoutapally, Gannavaram mandal, Andhra Pradesh, India

Abstract

Purpose: *Pseudomonas aeruginosa* is most frequently responsible for nosocomial infections **.**The emergence and the spread of Metallo-β-lactamases (MBLs) in *Pseudomonas aeruginosa* has created an alarming situation and has become a therapeutic challenge to clinicians and microbiologists. In this study, the phenotypic confirmation of the MBL production was done by different phenotypic methods.

Method: The 100 isolates of *Pseudomonas aeruginosa* were screened for Metallo-β-lactamase production by using Imipenem. All the Imipenem resistant isolates were further subjected to phenotypic confirmation of MBL by the combined disk test(CDT)using Imipenem with EDTA(Ethylene Diamine Tetraacetic Acid), the double disk synergy test(DDST) using Imipenem and EDTA and the modified Hodge test(MHT). The results were analyzed and tabulated.

Results: 20(20%) isolates of *P. aeruginoasa* were resistant to Imipenem, 15(75%) were positive for MBL production by CDT,8(40%) were positive by the DDST and 5(25%) were positive by the modified Hodge test . The prevalence of MBL production was 15%. MBL producing isolates showed widespread resistance to Cephalosporins, Aminoglycosides, Ciprofloxacin and Piperacillin with Tazobactum combination.

Conclusion: The results of our study showed that the combined disk test which used Imipenem, is more sensitive than the double disk synergy test and the modified Hodge test.The factors that support CDT as a feasible option are the ease of the performance, interpretation of the results and cost effectiveness. Hence, the routine testing of the Metallo- β-lactamases producers in limited resource settings by phenotypic methods is of great value, in order to take measures to advocate a proper antibiotic policy and also to take effective steps for controlling their spread worldwide**.**

KEYWORDS- MBLs, *Pseudomonas aeruginosa*, Imipenem-EDTA

INTRODUCTION

Pseudomonas aeruginosa is one of the leading causes of nosocomial infections and in the light of its numerous intrinsic and acquired mechanisms of drug resistance, it is a cause of concern for treating physicians. Although the antibiotic resistance in *P.aeruginosa* is caused by multiple mechanisms, one growing factor leading to resistance is the production of carbapenemases.[1]

The acquired Metallo-β-lactamases (MBLs) are carbapenemases which require zinc at the active site and are predominantly produced by *P. aeruginosa*. They belong to Ambler's Class B and Bush-Jacoby Mederios Group 3. In India only bla $_{VIM}$ and NDM-1 have been reported in *P. aeruginosa* .[1]

Metallo- Beta-Lactamases (MBLs) have recently emerged as one of the most problematic resistance mechanisms owing to their capacity to hydrolyse all beta lactams including carbapenems with exception of *Aztreonam*.[2]Acquired MBL genes are located on integron structures that reside on mobile genetic elements such as plasmids or transposons, thus enabling widespread dissemination. *Pseudomonas aeruginosa* producing metallo-β- lactamases was first reported from *Japan* in 1991 and then resistance spread to other species.[3]Several non molecular techniques have been studied, all taking advantage of zinc dependence of the enzymes by using chelating agents such as Ethylene Diamine Tetra Acetic acid(EDTA) or 2mercaptopropionic acid to inhibit their activities.A double disk synergy test(DDST) and combined disk test (CDT) have been reported as reliable methods for detection of MBLs in carbapenem-resistant *P.aeruginosa*. Genotypic methods are reliable and highly accurate but their accessibility is often limited to reference laboratories .[3] In India prevalence of MBLs production in *P.aeruginosa* varies from one region to another and range from 7 – 65%.[4] The present study was undertaken to know the prevalence of MBL producing *Pseudomonas aeruginosa* and to compare 3 phenotypic methods for detection of MBL namely Imipenem-EDTA combined disk test, Imipenem-EDTA double disk synergy test and Modified Hodge test at the tertiary care hospital, Vijayanagar Institute of Medical Sciences(VIMS), Bellary, Karnataka.

Materials and Methods

The study was conducted at the Department of Microbiology, Vijayanagar Institute of Medical Sciences (VIMS) .A total of 100 consecutive isolates of *P. aeruginosa* obtained from various clinical samples collected from patients admitted at VIMS hospital over a period of one year (January-December, 2011) were included in the study. All isolates were non-duplicate. The isolates were identified as *P.aeruginosa* by standard microbiological methods[5]. Imipenem sensitive isolates were excluded from the study.

Antimicrobial susceptibility:

Antimicrobial susceptibility of all the isolates was performed by the Kirby-Bauer disc diffusion method according to the CLSI guidelines.[6] The antibiotics tested were ,Cefotaxime (30 µg), Ceftazidime (30 µg), Cefoperazone (30 µg), Ceftriaxone (30 µg), Amikacin (30 µg), Ciprofloxacin (5µg), Gentamicin(10µg), (Piperacillin/ tazobactam (100 µg/10 µg) , Imipenem(10µg) and Polymyxin B(300 units) from Hi-Media Laboratories,Mumbai,India.

MBLscreening:

Screening for MBL production was done on Imipenem resistant isolates by the following methods:

Imipenem(IMP)-EDTA combined disc test:

The IMP-EDTA combined disk test was performed as described by Yong *et al* [7] .Test organisms were

inoculated on Mueller Hinton agar as recommended by the CLSI.[6] Two 10 µg imipenem disks were placed on the plate, and appropriate amounts of 10 µL of EDTA solution was added to one of the disks to obtain the desired concentration (750 µg). The inhibition zones of Imipenem and Imipenem-EDTA 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 Imipenem - EDTA disc was ≥ 7 mm than the Imipenem disc alone, it was considered as MBL positive.

Imipenem-EDTA double disc synergy test(DDST): This test was done as described by Lee et al[8].An imipenem (10 µg) disc was placed 20 mm centre to centre from a blank disc containing 10 µL of 0.5 M EDTA (750 µg). 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 drug was interpreted as a positive result.

Modified Hodge test(MHT)

This test has been originally described by CDC(Centre for Disease Control,Atlanta) for Carbapenemases detection in Enterobacteriaceae.[8]In this study this test was done for Carbapenemase detection in *P.aeruginosa* .

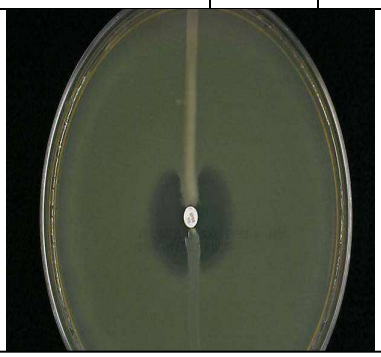
Procedure : A lawn culture of 1:10 dilution of 0.5 McFarland's standard *E.coli* ATCC 25922 broth was done on a Mueller Hinton agar plate. A 10µg Imipenem disk was placed in the centre of plate and 10µl of 50mM zinc sulphate solution was added to Imipenem disk, then Imipenem resistant *P.aeruginosa* were streaked from edge of the disk to the periphery of the plate in different directions. After overnight incubation, the plates were observed for presence of a cloverleaf shaped zone of inhibition and the plates with such zones were interpreted as positive for Modified Hodge test.

RESULTS

Out of the 100 isolates of *P.aeruginosa*, 64(64%) were from males and 36(36%) were from females. The number of samples were 32(32%),15(15%),18(18%),15(15%),12(12%)and 8(%) from wound swabs ,tracheal aspirate, urine, blood, sputum and other body fluids(CSF &pleural fluids) respectively. Imipenem resistance was observed in 20 isolates, out of these 15(75%) showed MBL positive by the 3 phenotypic methods and a comparison of these methods were shown in [Table1 and Fig-1,2&3]. The prevalence of MBL production was 15% of all the clinical isolates of *P.aeruginosa* and the % of MBL production in these samples were4/32(12.5%),4/15(26.6%),3/18(16.6%),2/15(13.3%),1/12(8.3%) and1/8(12.5%) from wound swabs ,tracheal aspirate, urine, blood, sputum and other body fluids(CSF &pleural fluids) respectively . Antibiotic resistance patterns in % for the 100 isolates were 70,70,68,68,40,38,56,20,25 and 02 for Cefotaxime,Ceftriaxone,Cefoperazone, Ceftazidime,Gentamicin,Amikacin,Ciprofloxacin,Imipenem,Piperacillin-Tazobactum and Polymyxin B respectively. Resistance patterns of MBL positive isolates in % were 100 for all the above Cephalosporins & Imipenem and 93.3,80,93.3,60 and 6.67 for Gentamicin, Amikacin, Ciprofloxacin,Piperacillin-Tazobactum and Polymyxin B respectively.

Table 1 -Comparison of MBL detection by phenotypic methods

Method	Samples tested	No of MBL detection	MBL detection %
MHT	20	5	25
CDST(I-EDTA)	20	15	75
DDST(I&EDTA)	20	8	40



Fig(1):**MHT**-Clover leaf shaped indentation

Fig(2):DDST- Enhanced zone of inhibition between Imipenem & EDTA

Fig(3):CDT-Zone of inhibition >7mm with Imipenem & EDTA.

DISCUSSION:

Extensive antimicrobial spectrum and firmness against most common β-lactamases, Imipenem generally represents one of the last alternatives for the treatment of nosocomial infections caused by multidrug-resistant gram-negative bacteria, particularly *P. aeruginosa*. However, the rapid spread of MBLs among major gram-negative pathogens, particularly *P. aeruginosa*, is an emerging threat and a matter of concern worldwide ; further, MBL-carrying bacteria are known to cause intractable nosocomial infections [9].

In this study resistance patterns of 100 isolates of *P.aeruginosa* correlates with study by Angadi KM et al[10]and maximum resistance was shown for Cephalosporins. 20(20%) isolates showed Imipenem resistance which was similar to study by Angadi KM et al[10] who showed Imipenem resistance to be 21.6% . Among Imipenem resistant isolates 75% showed MBL production which was consistent with the study by Varsha gupta et al [11] who demonstrated 69.5% of the MBL production.

Prevalence of MBL production in our study was 15% which was consistent with various studies by Deeba bashir et al [12]and B Behera et al [2] who showed prevalence of MBL production to be 11.66% and 39.56% respectively. The difference in prevalence can be attributed to conditions under which they are tested and heterogenous nature of MBLs.

In the evaluation of three selected MBL phenotypic assays (CDT, DDST, and MHT shown in Table 1). CDT was the most sensitive of the three (75%), followed by DDST(40%) and least was

MHT(25%).These results correlated with studies by B Behera et al [2] (CDT-88.8% and DDST-57.14%), and P Pandya et al[13](CDT-96.3%,DDST-81.48%) and differs from studies by Picao RC et al [14] at (SaoPaulo), Brazil during January –December 2005(CDT-80%,DDST-82.6%) who showed DDST using Ceftazidime & EDTA to be a more sensitive method than CDT.

The antibiotic resistance was high in MBL producing isolates when compared to overall resistance of 100 isolates. Among the tested antibiotics, only Polymyxin B remained a therapeutic option which showed 93.3% sensitivity in MBL positive isolates. The above result correlated with the study by P Pandya et al[13] and M shanti et al [15]who showed Polymyxin B sensitivity in MBL positive isolates to be 85.19% and 92% respectively.

CONCLUSION:MBL detection remains a divisive issue , clinical laboratories are in need of a simple and direct method to recognize such resistance in gram-negative bacteria especially in *Pseudomonas aeruginosa* to improve disease management. Furthermore, in recognition that MBL genotypes are not homogenous in geographical distribution, a generalized criteria for interpretation of MBL phenotypic assays may not be possible. Thus, it is recommended that the phenotypic assays should be assessed and adopted based on the local situation.

REFERENCES:

1.S Buchunde,DK mendriatta,V Deotale and P Narang.Comparison of disc and MIC reduction with polymerase chain reaction for detection of metallo-β-lactamase in *Pseudomonas aeruginosa*.Indian J Med Mirobiol.2012; 30(2):170-174.

2.B Behera,P Mathur ,A Das,A Kapil and V Sharma.An evaluation of four different phenotypic techniques for detection of metallobetalactamase produing *Pseudomonas aeruginosa*.Indian J Med Microbiol.2012;26(3):233-37.

3.Shobha KL,Lenka PR,Sharma MK,Ramahandra Land Bairy I.Metallobetalactamase production among *Pseudomonas* species and *Acinetobacter* species in coastal Karnataka. J Clin Diagn Res .2009;Oct13(3):1747-1753.

4.Dr Anil Rajput et al.Prevalence of Metallo-betalactamases producing *Pseudomonas aeruginosa*.Indian Journal of Basic and applied Medical Research,Sept 2012;1(4):304-308.

5.Goven JRW. *Pseudomonas, Stenotrophomonas, Burkholderia*, In: Collee JG, Marmion BP, Fraser AG ,Simmons A, editors. Mackie and MacCartney. Practical Medical Microbiology.14th ed.London:Churchil Livingston ;1996; 413-23.

6. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial disk tests ; Approved Standards , 9th ed. CLSI Document M2-A9 , Vol. 26 No.1 Wayne PA 2006.

7. Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, and Chong Y, “Imipenem-EDTA disk method for differentiation of metallo-*β*-lactamase-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp.,” *Journal of Clinical Microbiology* 2002; 40(10): 3798–3801.

8..Lee k,Lim YS,Yong D,Yum JH,Chong Y.Evaluation of Hodge test and imipenem-EDTA double disk synergy test for differentiation of metallo beta lactamases producing clinical isolates of *Pseudomonas and Acinetobacter spp*.J Clin Microbiol 2003;41:4623-9.

9. Kalantari E, Torabi V, Salimizand H, Soheili F, Beiranvand S, Soltan Dallal MM, First Survey of Metallo Beta Lactamases in Clinical Isolates of *Pseudomonas aeruginosa* at a Referral Burn Center in Kurdistan Province. *Jundishapur J Nat Pharm Prod*. 2012;**7**(1):23-6.

10. Angadi KM et al. Detection of antibiotic resistance in *Pseudomonas aeruginosa* isolates with special referene to Metallobetalactamases from a Tertiary care hospital in Western India.Int J Microbiol Res.2012;4(7):295-298.

11. Varsha gupta ,Shailpreet Sidhu and Jagadish Chander.Metallo-β-lactamase producing nonfermentative gram negative bacteria:An increasing clinical threat among hospitalized patients. Asian Pac J Trop Med. 2012 Sep;5(9):718-21.

12. Deeba Bashir et al.detaction of metallo-beta-lactamase (MBL)producing *Pseudomonas aeruginosa* at a tertiary care hospital in Kashmir.African Journal of Microbiology research.january 2011;5(2):164-172.

13. Nirav P Pandya et al.Evaluation of various methods for MBL detection in Gram negative bacilli.Int J Biol Med Ress.2011;2(3):775-777.

14. Picao RC et al.Metallo-beta-lactamase detetion:Comparitive evaluation of double disk synergy test versus combined disk tests for IMP-,GIM-,SIM-,SPM- and VIM-producing isolates.J Clin Microbiol.2008 Jun;46(6):2028-37.

15.M Shanthi and Uma Shekar.Multi drug resistant *Pseudomonas aeruginosa* and *Acinetobacter baumanii* infections among hospitalized patients:risk factors and outcomes.J Assoc Physicians India.2009;57:636-645