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ORIGINAL ARTICLE

Metallobetalactamase Producing Pseudomonas Aeruginosa: An Emerging Threat To Clinicians

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ABSTRACT

In recent years, carbapenem resistance due to the production of metallobetalactamase (MBL) in *Pseudomonas aeruginosa* and other Gram negative bacilli have been reported worldwide. Metallobetalactamase producing *P. aeruginosa* constitute nearly 20% of all nosocomial isolates. The present study was undertaken to detect the prevalence of metallobetalactamase producing *P. aeruginosa* in our hospital and to evaluate an easy, but specific test for the detection of metallobetalactamase production so that it would be feasible for our laboratory.

A total number of 140 *P. aeruginosa* strains which were isolated from different clinical specimens were studied. An antibiotic susceptibility test with antipseudomonal antibiotics was done as per CLSI guidelines. Imipenem resistant isolates were screened for carbapenem hydrolysis by the Hodge test and the modified Hodge test. Metallobetalactamase production was detected by the double disk synergy test (DDST) and the disk potentiation test.

Out of 140 *P. aeruginosa* strains, 18 (12.9%) were imipenem resistant. Amongst these 18 strains, 16(88.8%) were Hodge test and modified Hodge test positive and all 16 were found to be metallobetalactamase producers by the DDST and the disk potentiation tests.

We hereby conclude that the detection of metallobetalactamase producing *P. aeruginosa* strains by the disk potentiation test should be introduced in any clinical Microbiology laboratory in order to aid infection control.

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Introduction

Carbapenem resistance in Gram negative bacteria occurs mainly due to decreased outer membrane permeability, increased efflux pumps, alteration of penicillin binding proteins and carbapenem hydrolyzing enzymes i.e. carbapenemases. According to the Ambler scheme, carbapenemases can be classified into 3 molecular classes: class A (penicillinase), class B (metallobetalactamases i.e. MBL) and class D (oxacillinase)[1]. As per the Bush Jacoby-Mederios classification, the MBLs belong to Group 3[2].

The MBLs require one or more divalent cations, usually zinc, at the active site. The MBLs efficiently hydrolyse all beta lactams except aztreonam in vitro. The MBL activity blocking agents are EDTA, thiolcompounds and cupric chloride ($CuCl_2$), ferrous chloride ($FeCl_2$), etc.

The MBLs are not inhibited by clavulanic acid, sulbactum and tazobactum.[3]

Acquired or transmissible MBLs can be plasmid, integron or transposon mediated. The first MBL which was encoded on a plasmid, IMP-1 was discovered in Japan in 1988 [4]. Since then, the IMP-1 to 23 and the VIM-1 to 14 variants have been reported [5].

Because of their high antibiotic resistance and potential for rapid and generalized dissemination to other Gram negative bacteria, the early recognition of MBL producers is the need of the hour. Though several phenotypic and genotypic methods are available, no Clinical Laboratory Standard Institute (CLSI) guideline is available for the detection of MBL producers [6].

Hence, the present study was undertaken to determine the prevalence of MBL producing *P.aeruginosa* strains in our hospital and to evaluate an easy, cost effective and yet, specific phenotypic method for its detection.

Material And Methods

A total number of 140 *Pseudomonas aeruginosa* strains were isolated from different clinical specimens like pus and wound swabs, urine, sputum, blood, body fluids, endotracheal tube secretions, etc. in the Microbiology department and were identified as per the conventional methods [7].

The study was conducted from June 2008 to December 2009. The antibiotic sensitivity test was done by the disc diffusion method as per CLSI guidelines [8].

Screening of Imipenem resistant *P.aeruginosa* for carbapenem hydrolysis was done by The Hodge test [9] and the modified Hodge test [10]. Hodge test positive and modified Hodge test positive *P.aeruginosa* strains were tested for MBL production by the double disk synergy test (DDST) [10] and the disk potentiation test [11].

Hodge Test [9]

In this test, lawn culture was done on a Muller Hinton agar (MHA) plate with overnight broth culture of E.coli ATCC 25922; the opacity was adjusted to 0.5 McFarland's standard. Then, imipenem resistant *P. aeruginosa (*test strain) was inoculated by lawn culture on the same plate. A 10µg imipenem disc was placed in the centre of the plate. 10 µl of 50 mM zinc sulfate solution was added on the imipenem disk and the plates were incubated at 37ºC overnight. The presence of a distorted zone of inhibition was interpreted as a positive result for carbapenem hydrolysis screening by the Hodge test.

The Modified Hodge Test [10]

The modified Hodge test (MHT) has been originally described by the Centre for Disease Control (CDC) for Carbapenemases detection in Enterobacteriaceae; but we have included this test for carbapenemase detection in *P.aeruginosa,* with slight modification.

Principle of MHT: When the test strain produces the enzyme carbapenemase, it allows the growth of a carbapenem susceptible strain (E.coli ATCC 25922) towards a carbapenem disk. The positive result is a characteristic clover-leaf indentation.

Procedure: In MHT, a lawn culture of 1:10 dilution of 0.5 McFarland's standard E.coli ATCC 25922 broth was done on a Muller Hinton Agar plate. A 10 µg imipenem disk was placed in the centre of the plate and 10 µl of 50mM zinc sulfate solution was added to imipenem disk. Then, imipenem resistant P. aeruginosa (test strains) were streaked from the edge of the disk to the periphery of the plate in four different directions. After overnight incubation, the plates were observed for the presence of a 'clover-leaf' shaped zone of inhibition and the plates with such zones were interpreted as modified Hodge test positive.

The Double Disk Synergy Test [10]

An overnight broth culture of the test strain (opacity adjusted to 0.5 McFarland opacity standard) was inoculated on an MH agar plate. After drying, a 10µg Imipenem disk and a blank filter paper disk (6mm in diameter, Whartman filter paper no.2) were placed 10mm apart from edge to edge. 10 µl of 50mM zinc sulfate solution was added to the 10 µg imipenem disk. Then,

10µl of 0.5 M EDTA (Sigma, USA) solution was applied to the blank filter paper disk. After overnight incubation, the presence of an enlarged zone of inhibition towards the EDTA disk was interpreted as DDST positive.

THE DISK POTENTIATION TEST [11]

A lawn culture of the test strain was done on Mueller Hinton (MH) agar plates (opacity adjusted to 0.5 McFarland's standard). Two 10µg imipenem disks were placed on inoculated plates wide apart and 10 µl of 50mM zinc sulfate solution was added to each of the imipenem disks. Then, 5µl of 0.5M EDTA solution was added to one imipenem disk. After overnight incubation, an increase in zone size of ≥ 7mm around the Imipenem-EDTA disk as compared to the imipenem only disk was recorded as a positive result.

Results

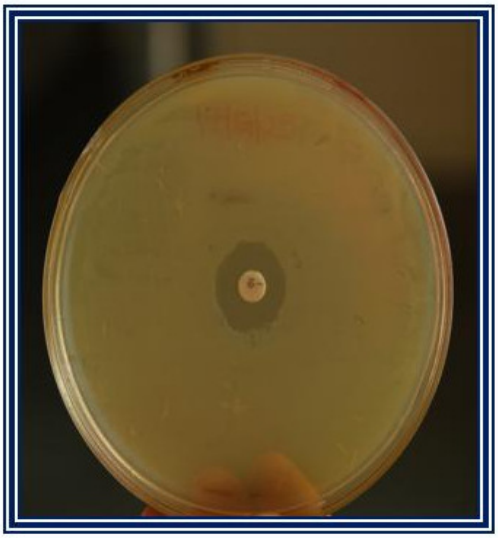
Out of the 140 *P. aeruginosa* strains studied 18(12.9%) were imipenem resistant and 16(11.4%) were MBL producers [Table/ Fig 1], [Table/ Fig 2] 18 imipenem resistant strains were screened for carbapenem hydrolysis by the Hodge test and the modified Hodge test (MHT) [Table/ Fig 3], [Table/ Fig 4] 16(11.4%) were positive for carbapenem hydrolysis and were also positive for MBL production by the DDST and the disc potentiation tests [Table/ Fig 5].

(Table/Fig 1) Prevalence of MBL producing *P. aeruginosa* strains

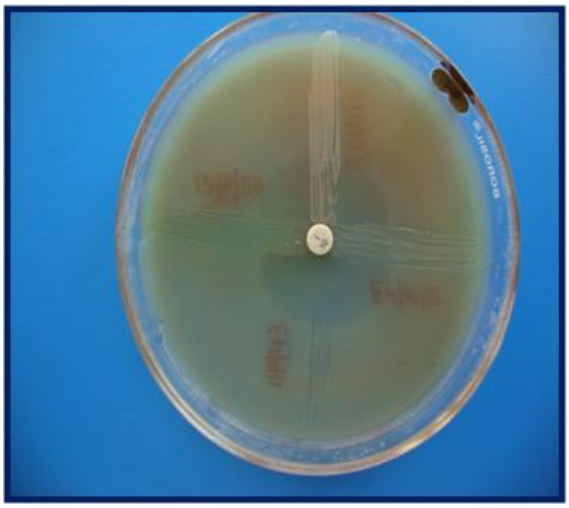
Total number of strains	Positive for MBLs	
	Numbers	percentage
n = 140	16	11.4

(Table/Fig 2) Detection of MBL in imipenem resistant *P. aeruginosa* strains (n = 18)

Carbapenem hydrolysis positive by		MBL production positive by	
Hodge test	Modified Hodge Test	DDST	Disk potentiation test
16	16	16	16



(Table/Fig 3) Hodge Test positive



(Table/Fig 4) Modified Hodge Test positive

(Table/Fig 5) DDST and Disk potentiation test positive

All (100%) *P.aeruginosa* strains were isolated from the indoor patients department (IPD) and 13(9.28%) were from the intensive care unit (ICU) patients. Out of 16 MBL producing *P.aeruginosa* strains, 8 (50%) were isolated from the Surgery ward, followed by 6 (37.5%) from the Medicine ICU department.

Out of the 16 MBL producing *P.aeruginosa* strains, 7(43.8%) were isolated from pus and wound swabs and 6 (37.5%) were isolated from urine [Table/ Fig 6]. All 6(100%) MBL producing *P.aeruginosa* strains isolated from urine samples, were from catheterized patients as compared to 12(46.1%) out of the 26 non MBL producing *P.aeruginosa* strains.

(Table/Fig 6) Isolation of MBL producing *P.aeruginosa* from different clinical specimens

Clinical specimens	*P. aeruginosa*		MBL positive *P. aeruginosa*	
	number	percentage	number	percentage
Pus & wound swab	40	28.6	7	43.7
Urine	32	22.9	6	37.5
sputum	35	25	1	6.2
Blood	17	12.1	0	0
Body fluids*	9	6.4	1	6.2
ET tube secretions	7	5	1	6.2
Total	140		16	

***Body fluids include: pleural fluid, ascitic fluid, synovial fluid & bronchial wash etc.**

Except polymyxin B, 15 (93.7%) MBL producing *P.aeruginosa* strains were resistant to all the 11 antibiotics studied as compared to only 2 (1.6 %) non MBL producing *P.aeruginosa* strains [Table/ Fig 7]. The highest sensitivity patterns observed among non MBL producing *P.aeruginosa* were for polymyxin B, imipenem and amikacin. All 140 (100%) *P. aeruginosa* strains including 16 MBL producing strains were sensitive to polymyxin B.

(Table/Fig 7) Antibiotic susceptibility pattern of MBL producing *P. aeruginosa* strains

Antibiotics	Susceptible strains			
	MBL positive (n = 16)		MBL negative (n = 124)	
	number	percentage	number	percentage
Imipenem	0	0	122	98.4
Piperacillin/tazobactam	0	0	80	64.5
Netillin	1	6.25	58	46.8
Ticarcillin	0	0	23	18.5
Amikacin	1	6.25	68	54.8
Ceftazidime	0	0	27	21.8
Ciprofloxacin	0	0	50	40.3
Gatifloxacin	0	0	50	40.3
Cefepime	0	0	8	6.5
Cefoxitin	0	0	8	6.5
Polymyxin B	16	100	124	100
Azotrenam	0	0	23	18.5

Discussion

Pseudomonas aeruginosa strains are intrinsically resistant to various antimicrobial agents. MBL producing *P.aeruginosa* is an emerging threat and a cause of concern for treating physicians. MBL have the ability to confer broad spectrum β-lactam resistance for a wide variety of antimicrobial agents which include the third generation cephalosporins, cephamycins and carbapenems, gentamicins and fluoroquinolones. The MBLs have become more notorious as therapeutically available inhibitors are not available and for their potential for rapid and generalized dissemination to different Gram negative bacilli, e.g. *P.aeruginosa*, *E.coli*, *Klebsiella pnemoniae*, *Acinetobacter spp.*, *Shigella flexneri,* etc.

Though PCR gives specific and accurate results, it's use is limited to only a few laboratories because of it's high cost and because of the different types of MBLs which are present worldwide[5].

In our study, we used the improved Hodge test with addition of 50mM of zinc sulfate on the imipenem disk as per Lee et al [9]. Similarly, in the present study, the modified Hodge test, the double disk synergy test and the disc potentiation test were also improved by us, with the addition of 10 µl of 50mM zinc sulfate solution to the imipenem disks. By using this modification, the interpretation of the test results were found to be more clear-cut, as metallobetalactamases require divalent cations at the active site for their activation, usually zinc.

The results of the improved Hodge test were compared with those of the modified Hodge test (MHT). Though the results of the improved Hodge test and the Modified Hodge test were similar on MH agar plates, the interpretation of the MHT was quite easy and in a same MH plate, 4 different *P. aeruginosa* strains could be tested.

Arkawa et al recommended the testing of the ceftazidime-resistant isolates for MBL production, because in their study, some MBL producing Gram negative bacilli were inhibited by the low concentrations of imipenem and they were difficult to detect [12]. But Lee et al reported that in their study, not a single MBL-producing isolate was detected among the imipenem susceptible isolates. [10].

Even in Japan, Sugino et al used only carbapenem resistant isolates for the screening of MBLs [13]. Hence, we also used carbapenem resistant isolates for the detection of MBL. Though Arkawa et al and other authors have done the DDST and the Disc potentiation tests with ceftazidime and EDTA, in our study, we used imipenem and EDTA for the DDST and the Disc potentiation tests. As in our study, even in non MBL producing *P.aeruginnosa* strains, the ceftazidime resistance was quite high (78.2%). The MBL producing strains may also have another ceftazidime resistance mechanism [9]. With such types of strains, DDST tests using an imipenem disc can show positive results for MBL, but a ceftazidime disc cannot; just as a cefepime disc but not a ceftazidime disc can detect extended spectrum β-lactamase (ESBL) production in Amp-C β-lactamase producing strains.

MBLs can hydrolyse all β-lactams except aztreonam in vitro [3] and therefore, we studied the utility of aztreonam (30µg) disc in MBL detection. Though Franklin et al have reported that 87% of their MBL producing Enteobacteriaceae isolates had >30 mm of zone with azotrenam, we did not find any MBL producing *P.aeruginosa* strain which was susceptible to aztreonam. This can only be explained by the fact that there is a presence of some other mechanisms for aztreonam resistance. Both a DDST and a disc potentiation test were also performed concurrently on a single MH plate.

In our study, the prevalence of MBL producing *P. aeruginosa* strains was 11.4%; which is similar to the studies conducted by Navneeth et al (12%) [14], Mendirattta et al (8.2%) [15], Hemlata et al (14%) [16] and Agrawal et al (8.05%) [17], respectively, from different parts of India.

In the present study, 2 imipenem resistant strains were found to be carbapenem hydrolysis negative by the Hodge test and MHT and the likely reason for the imipenem resistance may be a mechanism other than carbapenem hydrolysis, such as decreased membrane permeability [18].

As PCR can not be done in every laboratory, because of it's high cost, the disc potentiation test for MBL detection should be introduced in any routine Microbiology laboratory for effective infection control and to prevent therapeutic failure. The Disc potentiation test is easy to perform, is cost effective and quite specific amongst other phenotypic methods which are used for MBL detection. The Disc potentiation test can also be done along with routine antibiotic sensitivity tests.

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