

## ORIGINAL ARTICLE

**Laboratory Identification of Metallo-beta-lactamase Producing Clinical Isolates of *Pseudomonas aeruginosa*: An Assessment of Different Phenotypic Methods**

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**Abstract:**

**Background:** Multidrug Resistant (MDR) *Pseudomonas aeruginosa*, an emerging superbug causing a wide spectrum of nosocomial infections. Carbapenem resistance among *P. aeruginosa* isolates is of grave concern and is mainly due to production of Metallo-beta-lactamase (MBL) enzymes. **Aim and Objectives:** To find out the prevalence of MBL producing *P. aeruginosa* isolates from various clinical samples and to evaluate the efficacy of different phenotypic tests employed *in vitro* for their detection. **Material and Methods:** A total of 358 *P. aeruginosa* strains obtained from different clinical samples were subjected to antimicrobial susceptibility testing by Kirby-Bauer's Disc Diffusion method. All the imipenem resistant isolates were further tested by Modified Hodge Test (MHT) to detect carbapenem resistance. MBL production was tested by screening with Combined Disc Test (CDT) and Double Disc Synergy Test (DDST). MBL production was confirmed by MBL E-test (Ab BioDisk). **Results:** Among 358 strains of *P. aeruginosa* recovered, 114 isolates showed resistance to imipenem. E test demonstrated 73 out of 114 isolates as MBL producers. Of MHT, CDT and DDST tests performed, DDST showed high sensitivity and specificity. **Conclusion:** DDST can be suggested as an economical option in place of expensive E test for routine screening of MDR *P. aeruginosa* isolates for MBL production in a clinical laboratory; which is crucial for planning better management protocols.

**Keywords:** Modified Hodge Test, Combined Disc Test, Double Disc Synergy Test, MBL E-test

**Introduction:**

*Pseudomonas aeruginosa* is an opportunistic nosocomial pathogen with a versatile drug resistance profile. This superbug carries intrinsic and acquired resistance traits to multiple classes of antimicrobials including beta-lactams, aminoglycosides and fluoroquinolones. Infections caused by multi drug resistant *P. aeruginosa* pose challenges in treatment and are associated with increased mortality and morbidity rates in hospitalised patients [1].

Carbapenems are the preferred drugs for treating serious infections caused by penicillin or cephalosporin resistant gram negative bacilli, particularly extended spectrum beta lactamase producers. However, extensive and irrational use of these drugs in clinical practice resulted in wide spread occurrence of carbapenem resistant gram negative bacterial infections [2]. Carbapenem resistance is frequently encountered in non-fermenting bacilli such as *Pseudomonas aeruginosa* and *Acinetobacter* species [3]. Common mechanisms implicated were production of carbapenemases (carbapenem hydrolysing enzymes) and lack of drug penetration (porin mutations/efflux pumps) [1].

Carbapenemases are plasmid mediated enzymes capable of hydrolysing all beta lactam group of antibiotics including carbapenems, except aztreonam. Molecular studies classify carbapenem hydrolysing enzymes in to four classes A, B, C and

D. Metallo-beta-lactamases (MBLs) belong to class B enzymes and require divalent cations as cofactors for their activity and can be inhibited by metal ion chelators like Ethylenediaminetetraacetic acid (EDTA) [4].

MBL producing isolates of *P. aeruginosa* emerged as a global threat since last decade. In recent years MBLs have spread from *P. aeruginosa* to other gram negative bacilli through transferable plasmids leading to the worldwide dissemination of their genes [5, 6]. Hence, the detection of MBL producers among gram negative bacilli particularly *P. aeruginosa* is crucial for optimal treatment of hospitalised and critically ill patients and to restrict spread of drug resistance.

Though genotypic detection of MBLs was found to be specific and reliable, they are expensive and of limited availability only to reference laboratories. Different studies have reported detection of MBLs by phenotypic methods like imipenem-EDTA combined disc test, double disc synergy test using imipenem and EDTA, E-test and modified hodge test [7-9]. These methods are based on the ability of metal chelators like EDTA and thiol-based compounds to suppress the activity of MBLs.

In this context, the present study was undertaken to detect the prevalence of MBL producing *P. aeruginosa* isolated from various clinical samples and to evaluate the accuracy of four different phenotypic methods employed in the detection of MBL producers among imipenem resistant strains of *P. aeruginosa*.

#### **Material and Methods:**

The present prospective study was carried out over a period of one year, from January to December 2015, in the Department of Microbiology, GSL Medical College, Rajahmundry, Andhra Pradesh.

This study included 358 isolates of *P. aeruginosa* recovered from various clinical samples of hospitalised patients belonging to all age groups and both sexes. Samples such as pus or wound swabs, urine, ear discharge, sputum, broncho-alveolar lavage, tracheal, bronchial aspirates, and blood were included in this study. High vaginal swabs were excluded. Ethical committee approval was obtained from the institution and informed verbal consent taken from study group subjects.

All the 358 strains of *P. aeruginosa* identified in the laboratory by conventional methods, were subjected to antimicrobial susceptibility testing by modified Kirby Bauer's disc diffusion method as per Clinical and Laboratory Standard Institute (CLSI) guidelines [10]. Hi-Media discs used were imipenem (10 mcg), piperacillin-tazobactam (100/10 mcg), amikacin (30 mcg), ciprofloxacin (5 mcg), cefepime (30 mcg), ceftazidime (30 mcg) and colistin (10 mcg). Out of 358 isolates, 114 were found to be resistant to imipenem (carbapenem). *P. aeruginosa* ATCC 27583 was used as quality control strain. All imipenem resistant strains were further tested for carbapenemase production using modified Hodge test; screened for MBL production by combined disc test and double disc diffusion test; confirmed for MBL production by E- test (Ab BioDisk).

#### **Metallo-beta-lactamase detection methods**

##### **Modified Hodge test:**

A lawn culture was prepared on Mueller-Hinton Agar (MHA) using 1:10 dilution of 0.5 McFarland's standard *Escherichia coli* ATCC 25922. In the centre of the plate a 10 mcg imipenem disc (Hi Media) was placed. Test strains (imipenem resistant *Pseudomonas*) were heavily streaked from the edge of the imipenem disc to the periphery of the plate in four different directions.

The plates were incubated overnight and occurrence of clover leaf shaped zone of inhibition around the disc is considered MHT positive [11].

#### **Combined Disc Test or Disc Potentiation Test:**

A lawn culture of test strain with 0.5 McFarland's turbidity standard was made on MHA. Two imipenem discs were placed on the inoculated plate; one of it was impregnated with 10 µl solution of 0.5M EDTA. Following incubation for 16-18 hrs, an increase in the zone size of at least 7 mm around imipenem-EDTA disc compared to imipenem alone was read as MBL producer [12].

#### **Double Disc Synergy Test:**

An overnight broth culture of the test strain was inoculated on the MHA plate and dried. A 10 mcg imipenem disc and a sterile blank disc (Hi Media) were placed 10 mm apart from edge to edge. Another imipenem disc was placed far as control. The blank disc was impregnated with 10 µl of 0.5 M EDTA solution. Following overnight incubation, an expanded zone of inhibition around imipenem disc towards EDTA disc, compared to imipenem disc on the far side was interpreted as positive for MBL production [13].

#### **MBLE-Test:**

A MBL E-test strip (Ab BioDisk, Solna, Sweden) contains a double sided seven-dilution range of imipenem (4-256 mcg/ml) and imipenem (1-64 mcg/ml) in combination with a fixed concentration of EDTA. A lawn culture of test strain of 0.5 McFarland's opacity standard was prepared on MHA. E-Test strip was placed on the surface of agar. Following overnight incubation the plates were read for imipenem and imipenem- EDTA Minimum Inhibitory Concentration (MIC) values. When the MIC ratio of imipenem/imipenem+ EDTA was more than 8, the test was considered as MBL positive. Presence of phantom zone or the deformation of imipenem ellipse was also considered as positive [14].

#### **Results:**

Out of 358 isolates of *P. aeruginosa* obtained from various clinical samples, 114 were found to be imipenem resistant (zone of inhibition is 13mm or less) by Kirby-Bauer's disc diffusion method. Sample-wise distribution of *P. aeruginosa* from various samples was shown in table-1.

**Table 1: Sample-wise Distribution of Clinical Isolates of *P. aeruginosa***

Clinical sample	Number of isolates
<b>Pus/wound</b>	135(38%)
<b>Urine</b>	51(14%)
<b>Ear discharge</b>	66(19%)
<b>Sputum/ BAL*/Tracheal aspirates</b>	98(27%)
<b>Blood</b>	8(2%)
<b>Total</b>	358

\*BAL – Bronchoalveolar lavage

A total of 73(64%) among 114 imipenem resistant *P. aeruginosa* strains were detected as MBL producers by MBL E-test. Overall prevalence of MBL producing *P. aeruginosa* was found to be 20.4% (73 out of 358) in the present study. Table-2 depicts the evaluation of three different methods for MBL detection considering E-test as standard reference method. None of the three methods (MHT, CDT, DDST) produced results that

matched confirmatory E-test. Considering MBL E-test as standard, true positives and negatives; false positives and negatives were calculated for different phenotypic tests (Table-2) and a comparative statistical assessment of sensitivity, specificity, positive predictive value, negative predictive value and accuracy was done (fig.1). DDST proved to be more reliable than other tests with higher sensitivity and specificity.

**Table 2: Correlation of Different Phenotypic Methods in MBL Detection with E-test as Standard**

Methods	Imipenem resistant strains (n=114)				Sensitivity (%)	Specificity (%)
	MBL positivity by E-test (n=73)		MBL negativity by E-test (n=41)			
	MBL positive	MBL negative	MBL positive	MBL negative		
MHT	44(60%)	29(40%)	10(24%)	31(76%)	60.27	75.6
CDT	68(93%)	5(7%)	5(12%)	36(88%)	93.15	87.8
DDST	71(97%)	2(3%)	1(2%)	40(98%)	97.26	97.56

MHT – Modified Hodge Test; CDT – Combined Disc Test; DDST – Double Disc Synergy Test



**Fig. 1: Comparison of Efficacy Indices for Different Phenotypic Methods in MBL Detection**

**Discussion:**

*P. aeruginosa* is an apotheosis of drug resistance. It displays all sorts of antimicrobial resistance mechanisms including reduced outer membrane permeability due to loss of porins; production of beta-lactamases belonging to class D oxacillinases, class B carbapenemases, extended spectrum beta lactamases of class A, enhanced activity of efflux pumps, production of amino-glycoside modifying enzymes, structural modifications of topoisomerases resulting in quinolone resistance [1, 15]. Simultaneous expression of these mechanisms resulting in MDR phenotypes is a major challenge in deciding treatment protocols [16].

Different studies have reported the use of phenotypic methods like imipenem EDTA combined disc test, double disc synergy test using imipenem and EDTA, modified hodge test and E-

test in *P. aeruginosa* for detection of metallo-beta-lactamases. All these methods are based on ability of metal chelators like EDTA and thiol-based compounds to suppress the activity of MBLs [9, 12, 13, 14, 17]. Regarding the choice amongst them, there is a variation in the experience of different authors (Table-3).

The rate of occurrence of MBL producers among *P. aeruginosa* isolates obtained from different clinical samples in our setting was found to be 20.4% (73 out of 358) by E-test. Our results correlate with other studies by Sachdeva *et al.* (18.37%), Varaiya *et al.* (20.8%) and Murugan *et al.* (18.37%) [14, 18, 19]. Some studies by Bhalerao *et al.* (45%) and Mihani *et al.* (41%) reported higher prevalence of MBL producers among *P. aeruginosa* [12, 20].

**Table 3: Phenotypic Methods for MBL Detection – Experience by Different Authors**

Authors	Tests employed for MBL detection	Conclusions
Bhalerao <i>et al.</i> (2010) Loni, India	Imipenem-EDTA CDT, EDTA disc potentiation using cephalosporins, DDST	CDT proved to be more sensitive over DDST and disc potentiation methods
Bashir <i>et al.</i> (2011) Kashmir, India	CDT, MIC of imipenem + EDTA combination by agar dilution, MBL E-test	CDT, MIC reduction and E-test were equally sensitive
Jhon and Balagurunathan (2011) Bangalore, India	Disk potentiation test, DDST, Modified Hodge test, MIC of Meropenem by Broth Microdilution	DDST is reliable and reproducible compared to all phenotypic tests
Bose <i>et al.</i> (2012) Loni, India	Hodge test, CDT, DDST, MBL E-test	DDST and MBL E-test were equally effective
Sachdeva <i>et al.</i> (2017) Jaipur, India	Hodge test, CDT, DDST, MBL E-test	DDST and MBL E-test were effective.

CDT-Combined Disc Test; DDST-Double Disc Synergy Test; MBL E-test- Metallo Beta Lactamase Epsilometer test; MIC – Minimum Inhibitory Concentration; EDTA – Ethylenediamine tetra acetic acid

Among imipenem resistant strains the prevalence of MBL positive *P. aeruginosa* was considerably high (64%). Out of 114 imipenem resistant strains, 41 were found to be MBL non-producers by E-test. This could be attributed to mechanisms of imipenem resistance other than metallo-beta-lactamase production such as loss of membrane porins resulting in decreased permeability to the drug or active efflux pumps expelling the drug out of the bacterial cell [21-23].

MBL E-test detected 73 of 114 imipenem resistant strains as MBL producers. None of the other methods showed results similar to the E-test. Many studies have proven the superiority of E-test over other phenotypic methods [24, 25]. This may be attributed to the ability of MBL E-test to detect both chromosomal and plasmid mediated MBLs while other phenotypic methods were confined to the detection of few MBLs like IMP-1 and VIM enzymes in *P. aeruginosa* [25]. All the strains positive for MBL production by E-test showed MIC more than 16 mcg/ml. MBL E-test was reported to show 100% accurate results as with PCR in MBL detection by Khosravi *et al.* [26]. However E-test was found to be insensitive in detecting carbapenem sensitive MBL producers [27]. This may not affect results of this study as only carbapenem resistant isolates were screened for MBL detection.

Of the 73 MBL positive isolates by E-test, MHT could pick 44(60%), CDT and DDST could pick 68(93%) and 71(97%) respectively. False positives read by MHT were more (10) compared to CDT (5) and DDST (1). MHT was used for carbapenemase detection and cannot distinguish MBLs from non-MBL carbapenemases. These

false positives may represent imipenem resistant strains with mechanisms other than MBL production which needs further evaluation by genotyping methods.

Comparative assessment of three tests against E-test for sensitivity, specificity, positive predictive value, negative predictive value and accuracy was shown in fig.1. CDT with sensitivity and specificity of 93.15% and 87.8% respectively proved better than MHT (60.27% and 75.6%). DDST was found to be superior over CDT with higher sensitivity (97.26%) and specificity (97.56%). Our results were consistent with studies by Bose *et al.* and Sachdeva *et al.* [9, 14, 28].

The present study revealed E-test followed by DDST as effective methods for MBL detection. Hence, it is suggestible to routinely screen all imipenem resistant strains among multidrug resistant *P. aeruginosa* by DDST as it is both affordable and feasible to perform and interpret.

#### **Conclusion:**

MBL detection in *P. aeruginosa* isolates is crucial in planning for appropriate treatment and adequate infection control policies. E-test though sensitive could not be routinely performed in most resource poor setting laboratories due to cost constraints. Hence use of DDST in clinical laboratory would be a cost effective option for monitoring MBL resistant strains in gram negative bacilli.

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