# Evaluate the efficacy of real time pcr for MBL detection in phenotypically confirmed clinical isolates of pseudomonas aeruginosa in various clinical samples in a Tertiary Care Hospital

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**Abstract: CONTEXT: Carbapenems are the primary choice of treatment for severe *Pseudomonas aeruginosa* infection. However, the emergence of carbapenem resistance due to the production of Metallo-*β*-lactamases (MBLs) is of global concern.**
**MATERIALS AND METHODS: A total 200 clinical isolates of *Pseudomonas aeruginosa* were tested for MBL production. Meropenem (MRP) resistant *Pseudomonas aeruginosa* were taken as MBL screening. MBL detection was done by three phenotypic methods 1) Combined disk synergy test (CDST). 2) Double disk synergy test (DDST) and 3) E-test. The real time pcr test was used for detection of two gene encoding MBLs (IMP & VIM).**
**RESULTS: out of 200 *Pseudomonas aeruginosa*, 73 were resistant to Meropenem as screening positive. Out 73, 34(46.5%) isolates were MBL positive by CDST methods, 33(45%) by DDST method and 33(45%) by E-test. All 33 E-test positive strains contained the MBL gene where $bla_{VIM}$ and $bla_{IMP}$ gene were 23 and 10 respectively.**
**CONCLUSION: The study show that the high prevalence of *Pseudomonas aeruginosa* producing MBL enzymes thus the proper antibiotics policy and all carbapenems resistant *P. aeruginosa* should be routinely screened for MBL production using phenotypic methods and that Real time-PCR confirmation be performed to minimize the emergence of this MBL producing pathogens.**

**Keywords: Pseudomonas aeruginosa, Metallo-β-lactamase, CDST, DDST and E-test, $bla_{VIM}$ and $bla_{IMP}$ gene.**

## Introduction

Pseudomonas aeruginosa is a major source of opportunistic human pathogen most commonly isolated from gram-negative bacterial infection found in human worldwide. In spite of upgrading in antibiotic treatment, Pseudomonas aeruginosa is intrinsically resistant to a number of antimicrobial agents[1]. It is ubiquitous in nature mainly present as a saprophyte in warm moist conditions in the aqueous and environment sources including sinks, drains, respirators, humidifiers and disinfectant solutions.

Metallo-β lactamases (MBL) have recently emerged as one of the most worrisome resistance mechanism owing to their capacity to hydrolyze with the exception of Aztreonam, all β-lactam including carbapenem and also because their genes are carried on highly mobile elements, allowing easy dissemination. Such strains are not susceptible to therapeutic serine β lactamase inhibitors[2].
MBLs spread easily via plasmids and cause nosocomial infections and outbreaks. Such infections mainly concern patients admitted to the intensive care units with several comorbidities.[3] Therefore, early detection and identification of MBL producing organisms are of crucial importance for the prevention of nosocomial infection through appropriate treatment[4].
Detection of genes coding for MBL by polymerase chain reaction (PCR) usually gives reliable and satisfactory results; however, because of the cost the method, it is of limited practical use for routine diagnostic microbiology laboratories. Thus, a simple and inexpensive testing method for detection of MBL producers is necessary5. But This present study was undertaken to Evaluate the efficacy of real time pcr for MBL detection in phenotypically confirmed clinical isolates of *pseudomonas aeruginosa* in various clinical samples.

## Materials and Methods

The present study was carried out in the department of Microbiology, Shri Shankaracharya Institute of Medical Sciences for a period of one year, total 200 *P. aeruginosa* isolated from various clinical samples like pus, urine, blood, body fluids, sputum, catheter tip etc. were included. The all-clinical isolates recovered from both Indoor patients and outdoor patients. All isolates were subjected to antibiotic susceptibility testing as per the clinical and laboratory Standard Institute guidelines[6].
Screening of Metallo beta lactamase (MBL) was done by Modified carbapenem inactivation methods(mCIM) and the Isolates which gave MBL screening test positive were subjected to confirmation by three phenotypic methods.

### Antimicrobial Susceptibility testing

Antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion method as per CLSI guidelines[6]. In the present study the susceptibility testing was carried out against the following antibiotics: - ceftazidime(30µg), cefepime(30µg), ceftriaxone(30µg), cefoxitin(30µg), imipenem(10µg), meropenem(10µg), piperacillin/tazobactam(100/10µg), ticarcillin/clavulanic acid(100/75µg), aztreonam(30µg), gentamycin(10µg), amikacin(10µg), netilmicin(30µg), polymyxin B(300units), colistin(10µg).

in case of urinary isolates, ofloxacin(5µg) and norfloxacin(10µg) were also included. *P. aeruginosa* ATCC 27853 strain was employed as the control strain[7].

**Phenotypic methods used for confirmation of MBL production.**

**Imipenem (IMP)-EDTA combined disc test (CDST)**: - The IMP-EDTA combined disk test were performed as described by Yong et al. Test organisms were inoculated on to plates with Mueller Hinton agar as recommended by the CLSI. A 0.5 M EDTA solution was prepared by dissolving 18.61g in 100ml of distilled water and adjusting pH to 8.0 by using NaoH. The mixture was sterilized by autoclaving. Two 10µg imipenem disks (Becton Dickinson) were placed on the plate, and appropriate amounts of 10µl of EDTA solution were added to one of them to obtain the desired concentration (750µg). The inhibition zones of the Imipenem and Imipenem-EDTA disks 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 and EDTA disc will ≥ 7mm than the Imipenem disc alone, it will be considered as MBL positive[8].

**Double Disk Synergy Test (DDST):-** This test was performed with an overnight broth culture of the test strain inoculated on the MHA plate and allowed to dry. 10µl of the 0.5M EDTA solution was added to a 6mm blank filter paper disk (Whatman filter paper no.1) which contained approximately 750µg of EDTA. A 10µg imipenem disk was placed in the Centre of the plate flanked by EDTA disk at a distance of 20 mm Centre to Centre from blank disc containing 10µl of 0.5M EDTA (750µg). After overnight incubation, the presence of an enlarged zone of inhibition towards the EDTA disk was interpreted positive for an MBL producer[9].

**E-Test:** E-test metallo-beta-lactamase strips consisted of a double sided seven dilution range of imipenem IP (4 to 256µg/ml) and IP (1 to 64µg/ml) overlaid with a constant gradient of EDTA. Individual colonies were picked from overnight agar plates and suspended in 0.85% saline to a turbidity of 0.5 McFarland's standard. A sterile cotton swab was dipped into the inoculums suspension, and lawn culture of inoculums was done on MHA plate. The excess moisture was allowed to be absorbed for about 15 min before the E-test MBL (AB bioMerieux) strip was applied. Plates were incubated for 16 to 18 h at 37°C. The MIC end points were read where the inhibition ellipses intersected the strip. A reduction of imipenem MIC=3 two folds in the presence of EDTA will be interpreted as being suggestive of MBL production[10].

**Real time PCR for detection of genotypic identification of Metallo-beta-lactamases genes.**

**STEPS:**

a) DNA extraction from bacterial colony.
b) Master mix preparation
c) Template addition
d) DNA amplification in thermal cycler (Quant studio3).

**DNA extraction**:

DNA extraction was done by using silica spin column-based method using purification of genomic DNAfrom bacterial cells (chromous Biotech™) kit.

**Table: Equipment and Reagents Required: for DNA extraction.**

| Component | Concentration | 50 isolations | 250 isolations |
|---|---|---|---|
| Bacterial gDNA suspension Buffer | 5X | 10ml | 50ml |
| Bacterial gDNA lysis Buffer | 1X | 50ml | 250ml |
| RNaseA (DNase free, lyophilized) | - | 2 no | 10 no |
| Wash Buffer | 5X | 11 ml | 55 ml |
| Elution buffer | 1X | 10 ml | 50 ml |
| Spin column<br>Collection tube | -<br>- | 50 no.<br>50 nos. | 250 nos.<br>250 nos. |

a. Dilute 5X Bacterial gDNA suspension Buffer in 1X autoclaved distilled water (ChromousBiotech™).
b. Diluted 5X was washed Buffer with 1X absolute ethanol (ChromousBiotech™).
c. RNase was stored at 4ºC (ChromousBiotech™).
d. Absolute ethanol (100%).
e. RNase was stored at 4°C.
f. Absolute Ethanol (100%)
g. Distilled water was autoclaved
h. 1.5 ml or 2 ml microcentrifuge tubes
i. Finally, it was microcentrifuge.
j. Followed by water bath set at 65°C

**Preparation of RNASE solution**

a) 150µl of autoclaved distilled water was added into RNase vial, mixed by tapping and inoculated at 65ºC for 10-15 minute for efficient solubilization of RNase.
b) The concentration of RNase is 10mg/ml. Store RNase solution at 4ºC.

**Amplification**

The presence of *bla*IMP, *bla*VIM were tested in all E-test test positive isolates. The primers sequence used as fallows:

| The primer sequence used in the study | | | | |
|---|---|---|---|---|
| Target gene | Primer sequence | | Amplification in size | Reference |
| | Forward | Reverse | | |
| IMP | GTTTATGTTCATACWT CG | GGTTTAAYAAAACAACCAC | 432 | 11 |
| VIM | TTTGGTCGCATATCGC AACG | CCATTCAGCCAGATCGGC AT | 500 | 11 |

Polymerase chain reaction was carried out in 50 µl reaction mixture containing 5µl template, 1µl forward primer,1µl reverse primer,25µl PCR buffer(containing dNTPs, Taq polymerase).Samples were then subjected to initial denaturation at 950 C for 3 minutes, followed by 30 cycles of 950C for 30 seconds, 450C for 1 minute for VIM and 660C for IMP gene,720C for 1 minute and a final extension at 720C for 10 minutes to complete the elongation of the PCR intermediate products. For NDM amplification was carried out under the following thermal cycling conditions: 10 min at 94°C; 36 cycles of amplification consisting of 30 s at 94°C, 40 s at 52°C, and 50 s at 72°C; and 5 min at 72°C for the final extension

.PCR for all the 2 genes were done on all phenotypic confirmatory test positive isolates to find out the existence of single or multiple MBL genes in a single isolate.

**Result**

Out of 200 clinical isolates of *P. aeruginosa* 7(35%) were resistant to meropenem which were considered as MBL screening positive. These 73 isolates were studied for MBL detection by various phenotypic methods and real time pcr (Quant studio3)

| **Table 1: Gender and age distribution among the study subjects.** | | |
|---|---|---|
| **Gender** | **Number** | **%** |
| Male | 116 | 58 |
| Female | 84 | 42 |

Among 200 isolates Male were 116 and female were 84. **(Table1)**

Table 2: Age & sex wise distribution of different isolates.

| Age group (in year) | *Pseudomonas aeruginosa* isolated | | |
|---|---|---|---|
| | Male | Female | Total |
| 10-20 | 7 | 5 | 12 |
| 21-30 | 21 | 20 | 41 |
| 31-40 | 35 | 15 | 50 |
| 41-50 | 20 | 19 | 39 |
| 51-60 | 18 | 13 | 31 |
| 60-70 | 10 | 9 | 19 |
| >70 | 5 | 3 | 8 |
| Total | 116 | 84 | 200 |

Among 200 isolates, Majority were collected from male patients 116 and 84 isolates from female. The most of isolates of *Pseudomonas aeruginosa* were from the 21-30 age group patients (50) followed by patients from 41-50 age group (39). Whereas above 70 years age patient the isolates were 8. **(Table 2)**

Table 3: Age wise distribution of patients from the samples were obtained.

| Age group (in year) | *Pseudomonas aeruginosa* isolated | |
|---|---|---|
| | Total | Percentage |
| 10-20 | 12 | 6% |
| 21-30 | 41 | 20.5% |
| 31-40 | 50 | 25% |
| 41-50 | 39 | 19.5% |
| 51-60 | 31 | 16.5% |
| 60-70 | 19 | 9.5% |
| >70 | 8 | 4% |
| Total | 200 | 100% |

The isolates were from patients of various age groups, the age of the patients ranges from 10 years to 70 years. Maximum numbers of isolates 50(25%) were from the age group followed by 41(20.5%) from 21-30 age group, 39(19.5%) from 41-50 age group, 31(16.5%) from 51-60 age group, 19(9.5%) from 60-70 age group, 12(6%) from 10-20 age group & 8(4%) from above 70 years age. **(Table 3)**

Table 4: *Pseudomonas aeruginosa* isolates from different clinical samples.

| Clinical samples | No. of Isolates | percentage |
|---|---|---|
| Pus | 67 | 33.5% |
| Fluid | 45 | 22.5% |
| Urine | 36 | 18% |
| Endotracheal tube | 25 | 12.5% |
| Sputum | 15 | 7.5% |
| Blood | 7 | 3.5% |
| Folley's catheter tip | 5 | 2.5% |
| Total | 200 | 100 |

Out of 200 isolates from various clinical samples, the maximum were from pus 67(33.5%) followed by fluid 45(22.5%), Urine 36(18%), Endotracheal tube 25(12.5%), sputum 15(7.5%), blood 7(3.5%) and folley's catheter tip 5(2.5%). (**Table 4)**

**Table 4: comparison of 3 MBL phenotypic confirmatory methods.**

| | **IMP-IMP EDTA TEST** | **DOUBLE DISC SYNERGY TEST** | **E TEST** |
|---|---|---|---|
| Prevalence | 32.8% | 31.5% | 31.5% |
| Sensitivity | 95.6% | 100% | 100% |
| Specificity | 98% | 100% | 100% |
| P value | | | |

Among the three tests, the overall percentage of MBL production was 46.5% that the CDST was observed in 34 isolates whereas 33 isolates showed MBL activity by remaining two tests performed including DDST & E test.(Table:) Table shows the compression of screening and confirmatory tests in phenotypic detection of MBL. (Table 4)

33 isolates were positive for MBL production by CDST. Out of them 1 isolates showed false positive and 1 isolates were false negative. Whereas in E test and DDST all isolates were true positive and no isolates were found false negative. ( Table 5).

**Table 5 : confirmation of MBL by different phenotypic methods in screening positive isolates.**

| **Organisms** | **No. of screening positive isolates** | **Imp-EDTA combined disc test** | **Double Disc Synergy test** | **E test** |
|---|---|---|---|---|
| *Pseudomonas aeruginosa* | 73 | 34<br>Sensitivity=97%<br>Specificity=98 | 33<br>Sensitivity=100%<br>Specificity=100% | 33<br><br>Sensitivity=100%<br>Specificity=100% |

Table 6 : contingency table

| REAL TIME PCR | E TEST | | DDST | | CDST | |
|---|---|---|---|---|---|---|
| Positive= 33 | TP=33 | FN=0 | TP=33 | FN=0 | TP=33 | FN=1 |
| Negative = 50 | FP=0 | TN=40 | FP=0 | TN=40 | FP=1 | TN=39 |
| Total =73 | 33 | 40 | 33 | 40 | 34 | 40 |

**TP=TRUE POSITIVE, FP= FALSE POSITIVE, FN= FALSE NEGATIVE, TN= TRUE NEGATIVE**

33 isolates of *Pseudomonas aeruginosa* was MBL producer. The most of isolates of MBL were from 51-60 age group patients (11) followed by patients from 21-30 age group (9), patients from 41-50 age group (6). The MBL producer isolates were mostly isolated from male patients (20) as compare to female patients that were 13. No isolates was found to be MBL producer among age above 70 years. The association was not found to be statically significant. This concludes that there is no association between age group and sex with the above bacterial isolates their presence is just by chance (**Table 6).**

**Table 7: antibiotic resistant & sensitivity pattern among isolates**

| Antibiotic | Resistant | | Sensitive | |
|---|---|---|---|---|
| | Number | percentage | Number | Percentage |
| Amikacin | 132 | 66% | 68 | 34% |
| Aztreonam | 58 | 29% | 142 | 71% |
| Ceftazidime | 106 | 53% | 94 | 47% |
| Cefepime | 135 | 67.5% | 65 | 32.5% |
| Ceftriaxone | 133 | 66.5% | 67 | 33.5% |

| | | | | |
|---|---|---|---|---|
| Colistin | 0 | 0% | 200 | 100% |
| Gentamycin | 162 | 81% | 38 | 19% |
| Imipenem | 73 | 36.5% | 127 | 63.5% |
| Meropenem | 73 | 36.5% | 127 | 63.5% |
| ofloxacin | 162 | 81% | 38 | 19% |
| Pipracillin/Tazobactam | 105 | 52.5% | 95 | 47.5% |
| Polymyxin B | 0 | 0% | 200 | 100% |
| Ticarcillin/clavulanic acid | 67 | 33.5 % | 133 | 66.5% |

The Antibiotic sensitivity pattern amongst the isolates. There were 81% isolates that were resistant to ofloxacin and Gentamycin and 19% isolates that were sensitive respectively. There were 67.5% isolates were sensitive to cefepime and 32.5% were resistant. There were 66% isolates that were resistant to Amikacin and 34% isolates that were sensitive. All the samples were sensitive to Colistin & Polymyxin. There were 33.5% samples that were resistant to Ticarcillin/Clavulanic Acid and 66.5% samples that sensitive. There were 53% samples that were resistant to Ceftazidime and 47% samples that sensitive. There were 66.5% samples that were resistant to Ceftriaxone and 33.5% samples that sensitive and there were 100% isolates resistant to Tigecycline. **(Table 7)**

**Table 8: bla VIM and IMP detection among E test isolates by real time pcr.**

| Bla | N | % |
|---|---|---|
| VIM | 23 | 69.6 |
| IMP | 10 | 33.3 |

Demonstrated blaVIM & blaIMP detection among E-test isolates by real time pcr. There were 23(69.6%) isolates showed presence of blaVIM and there were 10(33.3%) isolates showed presence of blaIMP. (**Table 8)**

**Table 9: Correlation between genotypic and phenotypic methods**

| Variables | Value |
|---|---|
| r value | 0.54 |
| p value | 0.01 |

Correlation between genotypic and phenotypic methods showed that There was a statistically significant difference amongst the genotypic and phenotypic methods the p value was less than 0.01 and the r value came out to be 0.54**(Table 9)**

Table 10: antibiotics sensitive & resistant pattern for MBL (N=33)

| | Sensitive | Resistant |
|---|---|---|
| Amikacin | 25(76%) | 8(24%) |
| Aztreonam | 0(0%) | 0(100%) |
| Ceftazidime | 0(0%) | 33(100%) |
| Cefepime | 0(0%) | 33(100%) |
| Ceftriaxone | 0(0%) | 33(100%) |
| Colistin | 23(100%) | 0(0%) |
| Gentamycin | 15(43%) | 18(57%) |
| Imipenem | 0(0%) | 0(100%) |
| Meropenem | 0(0%) | 0(100%) |
| Ofloxacin | 10(30%) | 23(70%) |
| Pipracillin/Tazobactam | 13(39%) | 20(61%) |
| Polymyxin B | 33(100%) | 0(0%) |
| Ticarcillin/clavulanic acid | 22(66.6%) | 11(33.3%) |

All MBL producing isolates were resistant to Imipenem and meropenem by disc diffusion test. MBL producers also showed 100% resistant to Aztreonam,Ceftazidime,Cefepime,Ceftriaxone and all MBL isolates were sensitive to Colistin and PolymyxinB. Among other drugs Amikacin was founded to be the second most effective drug. **(Table 10)**

## Discussion

*Pseudomonas aeruginosa* is one of the commonly isolated organisms and it is becoming more resistant to commonly used antibiotics. Carbapenems and aminoglycosides were the two classes of drugs that showed be stativity against Pseudomonas. The frequency of MDR strains in Pseudomonas is also on the rise.

MBL production is a significant problem in hospital isolates of *P. aeruginosa*. With increasing isolation of ESBL-producing isolates in the hospital setting necessitating the use of carbapenems, the problem of MBL production is also increasing. The development

of simple screening tests designed to detect acquired MBL production is a crucial step towards large scale monitoring of these emerging resistant determinants. Though there are several screening methods recommended for the detection of MBL production, no single test when used alone is specifi c for these enzymes

A total 200 *P. aeruginosa* were isolated from various clinical samples such as pus, sputum, Urine, Catheter, blood etc. Out of 200 isolates from various clinical samples, the maximum were from pus 67(33.5%) followed by fluid 45(22.5%), Urine 36(18%), Endotracheal tube 25(12.5%), sputum 15(7.5%), blood 7(3.5%) and folley's catheter tip 5(2.5%). (**Table 4)**

In our study , all meropenem-resistant isolates had tested for MBL by different phenotypic methods and real time pcr for gene detection in phenotypically confirmed MBL but there are other resistance mechanism involved, such as permeability mutations via the loss of prions or the up-regulation of efflux system, which may be missed by Etest or by real time pcr. Previously reported novel VIM type of MBL producing *Pseudomonas spp*. detected in India during 2006 in which stains were clustered in 33 serotype with clone found in multiple hospitals.

Several types of VIM-2-carrying integrons were detected including a newly characterized variant, VIM-18 which showed a 4-amino-acid deletion compared to other VIM variants[12].

The present study shows that VIM and IMP type of MBL to be present among the isolates.

The data that was procured in this study suggests that MBL producing organisms almost always carry multiple resistance gene that leaves us with very limited therapeutic options for treatment. These genetic elements which harbor the multiple resistance genes are capable of getting transferred to other organisms posing a serious threat to the nosocomial infections. With strict infection control measures and judicious use of antibiotics the resistance towards carbapenems can be contained. The first step in checking the spread of MBL is its effective detection. Unfortunately, till date there are no guidelines for the detection. However the present study suggests various criteria for effective detection of MBL which if incorporated in routine diagnostic procedure will ensure effective treatment in patients.

The present study emphasizes the high prevalence of multidrug resistant *Pseudomonas aeruginosa* producing β-lactamase enzymes of diverse mechanisms. To combat these problems, epidemiological studies should be undertaken in hospital settings to monitor the source of infection. Early detection of these β- lactamase producing isolates in a routine laboratory could help to avoid treatment failure, as often the isolates producing this enzyme show a susceptible phenotype in routine susceptibility testing. Moreover, strict antibiotic policies should be implemented and disciplinary measures should be undertaken to limit the use of cephalosporins and carbapenems in the hospital settings. Otherwise, the continuous emergence of these multiple β-lactamase producing pathogen would leave no other option to treat Gram-negative nosocomial infections.

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