

Original Research Article

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Comparison of Different Phenotypic Tests for the Detection of Metallo-B-Lactamase (MBL) Producing *Pseudomonas aeruginosa*

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ABSTRACT

Pseudomonas aeruginosa producing Metallo-B-Lactamase (MBL) is emerging as a serious threat in the treatment of patients. Early and prompt detection of the same will help in timely implication of infection control measures and appropriate treatment. This study was taken up to compare the different phenotypic methods in the detect MBL producing *Pseudomonas aeruginosa* from pus samples. Only those isolates with pure and predominant growth of *P. aeruginosa* obtained from pus samples were included in the study. Four different phenotypic methods were used to test the MBL production in those isolates of *P. aeruginosa* which showed resistance to Imipenem – a) Imipenem (IMP) - EDTA combined disc test. b) Imipenem (IMP) - EDTA double disc synergy test. c) EDTA disc potentiation using Ceftazidime, Ceftizoxime and Cefotaxime. d) Modified Hodge test. A total of 1200 pus sample screened, of which 90 *P. aeruginosa* isolates were isolated. Out of the 90 *P. aeruginosa* isolated from pus samples, 66(73.3%) were Imipenem sensitive and 24(26.6%) were resistant. Of the 24 *P. aeruginosa* resistant to Imipenem, IMP-EDTA CDT detected 14(58%) of ESBL producers, IMP-EDTA DDST detected 7(29%), EDTA disc potentiation test detected 11(46%) and Modified Hodge method detected 8(33%). And 2 (8.33%) did not show MBL production property by any of the four methods used in the study. 100% sensitivity was noted to Colistin and Polymixin B in all the MBL harboring *P. aeruginosa*. Thus according to this study it was found that IMP- EDTA combined disc test has better detection rate of MBL producing *P. aeruginosa*, which can be easily incorporated in routine laboratory procedures.

Keywords

Pseudomonas aeruginosa,
Imipenem
resistance, Metallo-
B-Lactamase
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Introduction

Due to the wide use of β - lactams especially the carbapenems in the treatment of beta-lactam resistant bacteria including ESBL producing bacteria, MBL producing organisms have increased in number and are posing a serious problem in treating them.¹

MBL production has been increasingly reported among *Pseudomonas aeruginosa*, which is an important nosocomial multidrug resistant pathogen.^{2,3} Metallo beta lactamase (MBL) belongs to Ambler class B enzymes.

With the help of divalent cation zinc as cofactor for enzymatic activity, they hydrolyze carbapenem as-well-as other β -lactam antibiotics. The substrate spectrum of this enzyme is quite broad, as it can hydrolyze penicillins, cephalosporins and carbapenems, but it lacks the ability to hydrolyze aztreonam.⁴ Genes responsible for MBL production are IMP and VIM and they are coded by plasmids which in turn are involved in transfer of resistance from one bacteria to another.⁵ Chromosomal MBL was first

detected in environmental and opportunistic pathogenic bacteria such as *Bacillus cereus*, *Aeromonas* spp., and *Stenotrophomonas maltophilia*. Lately, there has been a dramatic increase in the detection and spread of acquired and transferable families of these metallo-enzymes (IMP, VIM, SPM, GIM, SIM and AIM enzymes).⁴In 1991, Japan reported the first case of *Pseudomonas aeruginosa* producing and from then it has been reported in various parts worldwide.⁶

CLSI guidelines don't mention about the standard available and reliable method for detection of these enzymes in *P. aeruginosa*,⁷

This study was taken up, to compare different phenotypic tests for identification of producing *P. aeruginosa* from pus samples.

This possibly will help to prevent the associated morbidity and mortality caused due to this organism by implementing proper control measures and to evaluate antibiotic therapy.

Materials and Methods

A total of 1200 pus samples were screened in one year which were received at the Department of Microbiology, J. N. Medical College, KLE university, from hospitalized patients of K.L.E.'S DR. Prabhakar Kore's Charitable Hospital and MRC, Belagavi were processed.

Isolates of *P. aeruginosa* obtained from pus samples as pure and predominant growth were considered for the study. Based on colony morphology and biochemical tests, organisms were identified. Using disc diffusion method, sensitivity of the isolates to imipenem/meropenem was determined using the control strain of *P. aeruginosa* ATCC 27853. CLSI guidelines were followed to interpret the results.

MBL production was tested only in those isolates of *P. aeruginosa* showing resistance to Imipenem, by the following methods

Imipenem (IMP) - EDTA combined disc test

Imipenem (IMP) - EDTA double disc synergy test

EDTA disc potentiation using ceftazidime, ceftizoxime and cefotaxime

Modified Hodge test.

Controls used For MBL tests⁸:

Positive control-*P. aeruginosa* ATCC 68549

Negative control-*P. aeruginosa* ATCC 27853

EDTA solution preparation

186.1 g of disodium EDTA.2H₂O was dissolved in 1000 ml of distilled water. pH 8 was adjusted using Sodium hydroxide (NaOH) and then autoclaving for sterilization. Each time 10µl of 0.5 M EDTA solution was used for the test. Solution prepared can be stored in airtight vials for 16weeks in refrigerator at 4⁰C or -20⁰C without significant loss of activity.⁹

MBL screening tests

Imipenem (IMP) - EDTA combined disc test¹⁰

Two 10mcg Imipenem discs are placed on the MHA plate which was prepared by lawn culture of standard inoculum (0.5 McFarland) of the test organism. To one of the IMP discs, 10µl of 0.5M EDTA solution was added to obtain a concentration of 750mcg and was incubated overnight at 37⁰C.

Isolate was considered as MBL producer if >7mm increase in inhibition zone with IMP-

EDTA disc compared to IMP disc alone was noted.

Imipenem (IMP) - EDTA double disc synergy test¹⁰

An IMP (10mcg) disc and a blank disc(6mm diameter, Whatmann No.2) containing 10mcl of 0.5 M EDTA(750mcg) were placed 20mm center to center on the MHA plate prepared with lawn culture of 0.5 McFarland suspension of the test organism and then was incubated overnight at 37°C.

Isolate was considered as MBL producer if any increased zone of inhibition in the area between IMP and EDTA disc in comparison with zone of inhibition on far side of the drug was seen.

EDTA disc potentiation using ceftazidime, ceftizoxime and cefotaxime¹⁰

A black disc containing 10mcl of 0.5 M EDTA (750mcg) solution was placed at the center of the plate and then the above three antibiotic discs were placed 25mm center to center from blank disc on the MHA plate prepared with lawn culture of 0.5 McFarland suspension of the test organism, followed by overnight incubation at 37°C.

Isolate was considered as MBL producer if any increase in the inhibition zone in the area between EDTA disc and any one of the three cephalosporin disc in comparison with zone of inhibition on far side of the drug was seen.

Modified Hodge test¹¹

MHA plate was lawn cultured with 0.5 McFarland of the E.coli ATCC 25922. Test organism is streaked across the plate to form a plus sign and the an IMP disc (10mcg) is placed at the center of the plate, followed by overnight incubation at 37°C.

Cloverleaf shaped inhibition is considered as a carbapenemases producer.

Results and Discussion

Out of 1200 pus sample screened, 90 *P. aeruginosa* isolates were isolated.

Out of the 90 *P. aeruginosa* isolated from pus samples, 66(73.3%) were Imipenem sensitive and 24(26.6%) were resistant.

Of the 24 *P. aeruginosa* resistant to imipenem, IMP-EDTA CDT detected 14(58%) of ESBL producers, IMP-EDTA DDST detected 7(29%), EDTA disc potentiation test detected 11(46%) and Modified Hodge method detected 8(33%). And 2 (8.33%) did not show MBL production property by any of the four methods used in the study (Figs 1–3).

Pseudomonas aeruginosa exhibits intrinsic resistance to several antimicrobial agents, thus becoming the leading cause for various nosocomial infections. However, acquired resistance to anti-pseudomonal β -lactams such as Piperacillin, Aztreonam, Cefepime, Ceftazidime, and Carbapenems considered as deterrent weapon that can be a major challenge in managing MDRPA infections, especially while it is associated with co-resistance with other classes of drugs namely Aminoglycosides and Fluoroquinolones.

Several mechanisms contributing to the acquired β -lactam resistance in *P. aeruginosa*. Few of them are-decreased outer membrane permeability, production of β -lactamases mainly MBL or the upregulation of efflux systems.

In the present study 26.6% (24/90) *P. aeruginosa* were resistant to Imipenem which is similar to study done by Shobha *et al.*,⁸ showing 30% of Imipenem resistant *P.*

aeruginosa. Another study done by Varaiya *et al.*,¹² showed 20.8% *P. aeruginosa* resistant to Imipenem. A study by John S *et al.*,¹³ showed 27.7%, Zahra *et al.*,¹⁴ showed 30.2%, Irfan *et al.*,¹⁵ showed 25% and Kumar *et al.*,¹⁶ showed 32.4% Imipenem resistant *P. aeruginosa*.

Study in few other places like Bangalore, by Navneeth *et al.*, found 12% of MBL producing *P. aeruginosa*.¹⁷ which is less in comparison to our study.

Variation in sample size studied or differences in hygienic practices may be the cause for the difference in the prevalence of MBL producing *P. aeruginosa* in different places/studies.

In the present study, IMP-EDTA CDT was a better method compared to other methods for detection of MBL producing *P. aeruginosa*, as in this study, of the 24 Imipenem resistant *P. aeruginosa*, Imipenem (IMP)- EDTA combined disc test detected 14(58%) and EDTA disc potentiation using Ceftazidime, Ceftizoxime and Cefotaxime which detected 11(46%), Modified Hodge test detected 8(33%) and Imipenem(IMP)- EDTA double disc synergy test detected 7(29%). This is in

accordance with a similar study by Behera *et al.*,¹⁰ showed CDT is superior to DDST and DPT for detection on MBL producing *P. aeruginosa*.

Another study by Yan JJ *et al.*,¹⁸ also showed that CDT is better method to detect MBL producing *P. aeruginosa*. Murugan *et al.*, showed DDST detected 70% and MHT detected 50% of MBL producing *P. aeruginosa* and hence showed DDST was more accurate than MHT in detecting MBL producing *P. aeruginosa*.¹⁹

Pragapati SB *et al.*,²⁰ showed DDST detected 16(27.11%) and CDT detected 30(50.84%) of MBL producers, which is similar to a study done by Boghiel *et al.*,²¹ MBL producer prevalence study done by Agrawal G *et al.* in clinical isolates of *Pseudomonas aeruginosa* using DDST and MIC methods using 174 isolates gave same results for both the combinations, and showed that MIC reduction is a cumbersome, laborious method. Thus found DDST to be the performed method.

And hence, DDST is recommended for detection of MBL production in *P. aeruginosa* as a screening method.²²

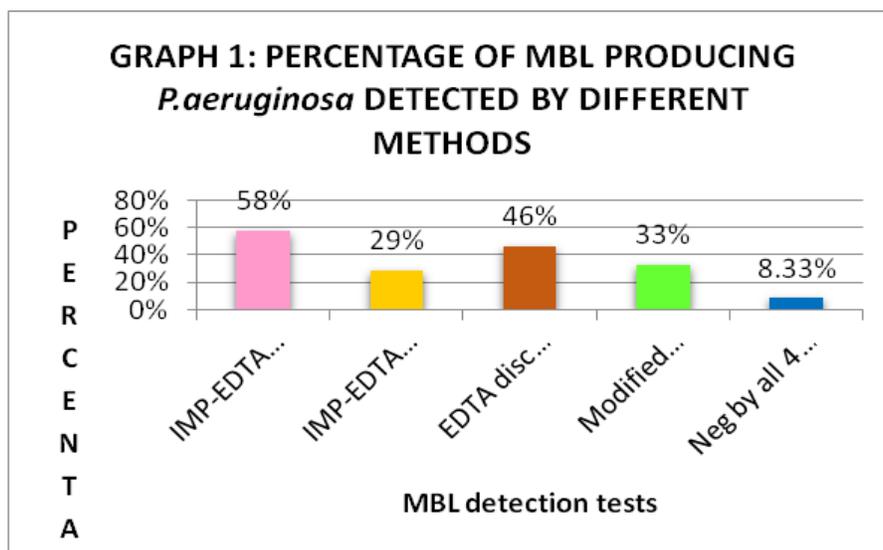


Fig.1 Imipenem (IMP)-EDTA combined disc test (IMP-EDTA CDT). Imipenem (IMP)-EDTA double disc synergy test (DDST)

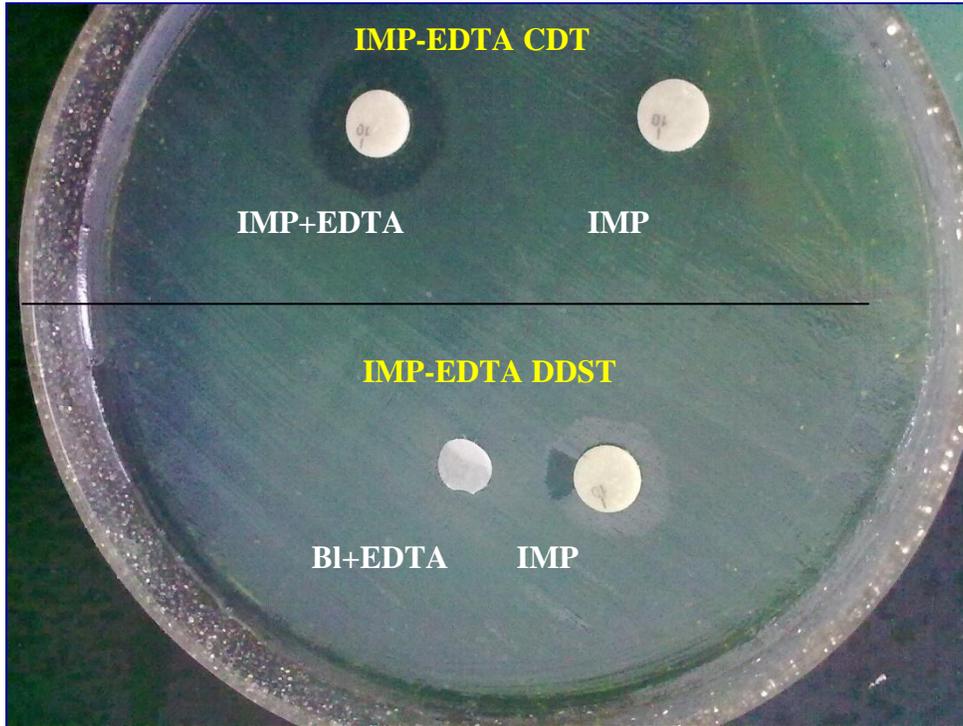


Fig.2 EDTA disc potentiation using Ceftazidime, Ceftizoxime, and Cefotaxime

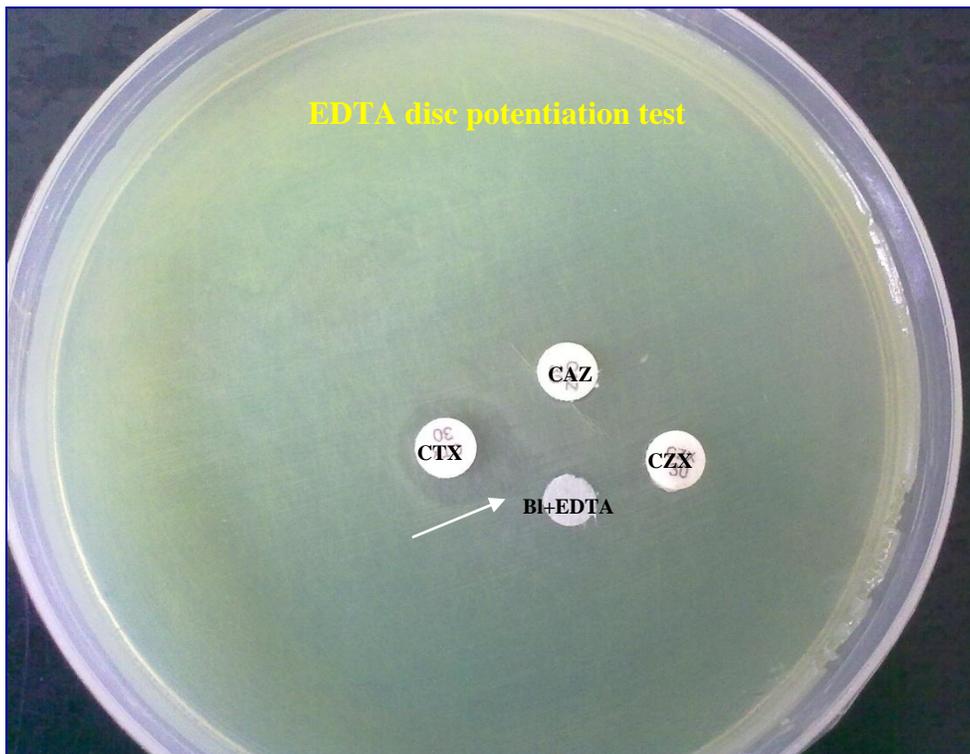
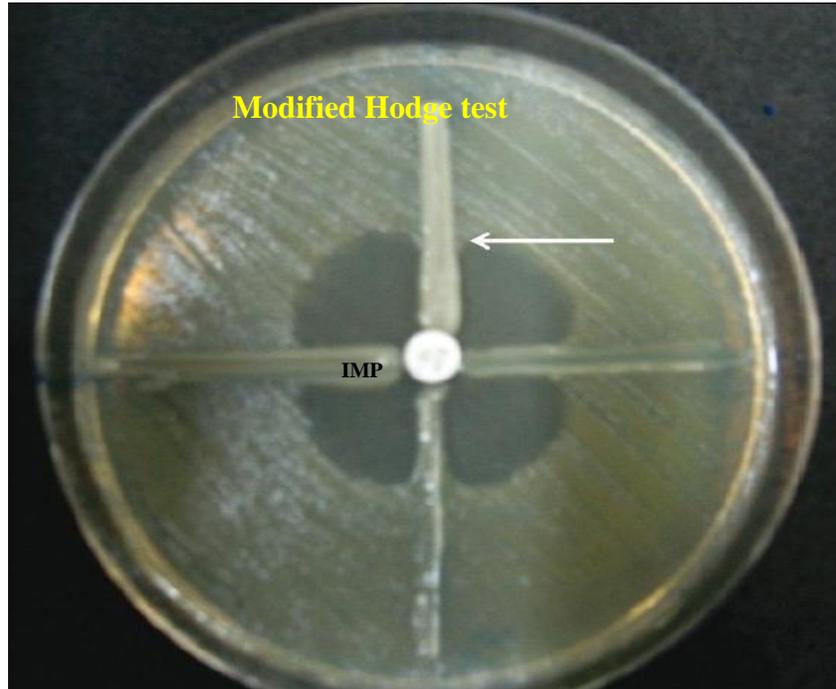


Fig.3 Modified Hodge test



A study by Noyal *et al.*, showed of the 18.9% Merapenem resistant, 16 (50.0%) were MBL producers by EDTA disk synergy test and 9 (28.1%) positive by modified Hodge test, and hence prepared method for MBL detection was eTDA disk synergy test over modified Hodge test.²³

Impermeability of outer membrane and or active efflux mechanism could be the possible reasons for the two (8.33%) Imepenim resistant *P. aeruginosa* which gave negative results for the detection of MBL producer by all the four methods used.

The main limitations of this study are, due to the absence of any standard methods to detect MBL in non-fermenters, it is difficult to comment on true or false MBL producers, MIC reduction would be a better method to know the drug susceptibility, but it is a cumbersome, laborious method and PCR could have been an additional investigation to detect the genes responsible for resistant, but has the disadvantage of its high cost.

Of the total 90 patients from whom *P. aeruginosa* were isolated, those which were not ESBL and MBL producers improved with treatment by third generation Cephalosporins, Erythromycin, Ampicillin and Ciprofloxacin ear drops. 3 patients were take-up for amputation, 3 of them expired due to development of septicemia and 12 patients could not be followed up.

All the patients in whom MBL producing *P. aeruginosa* were treated with colistin and polymixin B.

Thus according to this study it was found thatfor the detection of MBL producing *P. aeruginosa* Imipenem (IMP)- EDTA combined disc test is a best method.

Interpretation of IMP-EDTA DDST

Isolate is considered as MBL producer if >7mm increase inhibition zone size of with IMP-EDTA combined disccompared to IMP disc alone was noted.

Interpretation of IMP-EDTA CDT

Isolate is considered as MBL producer if, any increase in inhibition zone of IMP in the area between IMP and blank disc + EDTA disc when comparison far side inhibition zone of IMP drug noted.

Interpretation of EDTA disc potentiation test

Isolate is considered as MBL producer if, any increase in the inhibition zone in the area between blank disc+EDTA disc and CTX in comparison far side of drug inhibition zone noted.

Interpretation of Modified Hodge Test

Cloverleaf shaped zone of inhibition around the IMP disc

Increased occurrence of MBL producing *P. aeruginosa* in clinical strains is alarming and reflects the excessive use of carbapenem by the clinicians. Thus early and accurate detection of MBL producing *P. aeruginosa* has helped the doctors to treat the patients early with appropriate antibiotics, thereby improving the patient outcome and decreased the morbidity and mortality. And it also calls for a strict need of following antibiotic policy to avoid excess and inappropriate use of carbapenems.

Thus according to this study it was found that Imipenem (IMP) - EDTA combined disc test to be the preferred method for detection of MBL producing *P. aeruginosa*.

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